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Full Name of Author — Nom complet de l'auteur
ROMAN DANIEL HARDY GIETZ

Date of Birth — Date de naissance DEC 31, 1954	Country of Birth — Lieu de naissance CANADA

Permanent Address — Résidence fixe
10655-83 AVE APT 402
EDMONTON, ALBERTA
T6E 2E3

Title of Thesis — Titre de la thèse
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THE ANALYSIS OF DOPA DECARBOXYLASE EXPRESSION DURING EMBRYOGENESIS IN *DROSOPHILA MELANOGASTER*

by

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ROMAN DANIEL HARDY GIETZ

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DATED January 24 1984

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE ANALYSIS OF DOPA DECARBOXYLASE EXPRESSION DURING EMBRYOGENESIS IN *DROSOPHILA MELANOGASTER* submitted by ROMAN DANIEL HARDY GIETZ in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

R B Hodgetts

Supervisor

Frank Nargang

Verner Paetkau

J. F. Marsh

External Examiner

Date Dec 13, 1983

to Catherine and Jessica Lee

Abstract

Dopa decarboxylase (DDC) gene expression throughout embryogenesis in *Drosophila melanogaster* was investigated. A substantial rise in DDC activity was found during the late stages of embryogenesis, just prior to first instar larval hatching, and hours after embryonic ecdysteroid titer peak that occurs during mid-embryogenesis. This situation contrasts with the concurrent rise in DDC activity and ecdysteroid titer that occurs during pupariation suggesting that the relationship between the hormone and the onset of DDC activity during embryogenesis differs.

The possible reasons for the different temporal relationship between the embryonic DDC activity and the ecdysteroid peak were investigated. The embryonic ecdysteroids were found to be predominently 20-OH ecdysone and ecdysone, similar to those seen during pupariation. The embryonic DDC protein molecule was found to be similar, if not identical, to the puparial protein by genetic, immunological and *in vitro* translation studies. Thus the temporal lag between the embryonic hormone peak and the onset of DDC activity is possibly due to a different interaction of the two during this stage.

An examination of the RNA transcript pool throughout embryogenesis, using high resolution northern gel blot analysis, showed that DDC transcript accumulation occurs at two times, both distinct from the ecdysteroid titer peak. In the ovary and early embryos samples numerous DDC transcripts are seen, however these disappear by the third hour of embryonic development. DDC transcripts begin to accumulate again just prior to the DDC activity seen during late embryogenesis. This result shows that the temporal relationship between the embryonic ecdysteroid peak and the accumulation of DDC transcripts is similar to the late puffing response of the polytene chromosomes described by Ashburner (1974).

An initial characterization of the embryonic transcripts is presented. Nine transcripts of various sizes and steady state levels are visualized. A core of three transcripts are common to both early and late embryonic times, with some similarity between embryo transcripts and those seen at pupariation. The transcripts at each embryonic stage are characterized for size, homology to various DDC gene probes, and interaction with the cellular translation machinery. A model is proposed, in light of the results, outlining the production of mature translatable DDC mRNA from the primary gene transcript.

"Almost anything you do will be insignificant,
but it is very important that you do it."
Mohandas K. Gandhi

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I. INTRODUCTION

Regulation of gene expression is central to ontogeny and cellular differentiation in multicellular organisms. During development, differential expression of the genetic material reflects a cell's spatial and temporal identity within the organism (Davidson, 1976; Arking, 1978; Cullen *et al*, 1980; and Biessman, 1981). In order to understand the process of development the signals regulating gene expression must first be defined.

Recently, studies on the structure of eukaryotic genes and the events leading to gene expression have revealed that multiple levels of control exist. The molecular cloning of eukaryotic protein coding genes has shown that a large number of them have their coding regions interrupted by a variable number of non-protein coding intervening sequences (introns) (see Breathnach and Chambon, 1981 for review). Shortly after primary transcript initiation the 5' end is modified by the addition of a cap structure (Shatkin, 1976). Both intron and protein coding regions (exons) are copied into the primary gene transcript. Following transcription termination, the 3' end is also modified by the addition of a polyadenylate tail (Brawerman, 1981). Finally, the intron sequences are accurately spliced out to yield a translatable mRNA which is then exported from the nucleus (Nevins, 1983). Thus regulation of gene expression can and has been shown to occur at a number of different stages during the production of a gene product (for reviews see Darnell, 1982; Nevins, 1983). In this introduction, I shall outline the variety of levels at which the regulation of gene expression can occur in an attempt to gain some understanding of which levels are important to the ontogeny of an organism.

The most frequent method of regulating eukaryotic gene expression is at the level of transcript initiation. During the infection of certain eukaryotic cells with a DNA tumor virus (Adenovirus-2, SV-40, Polyoma) the differential temporal expression of the viral genome has been shown to be controlled by promoter selection and transcript initiation (see Ziff, 1980 for review). Positive regulation of transcript initiation has been shown for the metallothionein gene (Hager and Palmiter, 1981), the rat α2U globin gene (Kurtz, 1981), the chicken ovalbumin (Tsai *et al*, 1979), conalbumin (McKnight and Palmiter, 1979), and vitellogenin (Wiskocil *et al*, 1980) genes which are all responsive to hormone stimulation. Also the expression of the heat shock genes (Ashburner and Bonner, 1979) seems to be regulated at the transcriptional level. The chicken globin genes (Weintraub *et al*, 1981) represent developmentally regulated genes whose expression is also transcriptionally controlled. Thus, signals regulating gene transcription are important in the control of eukaryotic gene expression.

EUKARYOTIC PROMOTERS

The recent advances in defining the eukaryotic promoter have been made possible by recombinant DNA technology, *in vitro* or surrogate genetics and the accumulated knowledge about the functional domains of prokaryotic promoter elements (see Reznikoff and Abelson, 1978).

In most cloned eukaryotic genes, transcribed by RNA polymerase II, a region known as the TATA box resides 25 to 30 nucleotides upstream from the mRNA initiation site (see Breathnach and Chambon, 1981 for review). This sequence is in some ways related to the Pribnow box found at the -10 position in most prokaryotic promoters (Reznikoff and Abelson, 1978). The TATA box seems to be involved in the positioning of the mRNA start site (Benoist and Chambon, 1981) and is important for efficient *in vivo* transcription (Grosschedl and Birnstiel, 1980; Grosschedl *et al*, 1981; Grosveld *et al*, 1982; McKnight and Kingsbury, 1982). Sequences further upstream from the TATA box have also been shown to be important for *in vivo* transcription of cloned genes. The region about the -66 nucleotide is important in maintaining efficient transcription (Pelham, 1982; McKnight and Kingsbury, 1982; Mellon *et al*, 1981; Grosveld *et al*, 1982). Recently, Dierks *et al* (1983) have shown that a region positioned about the -100 nucleotide upstream from the mRNA cap site is also important for efficient *in vivo* transcription of a chimeric rabbit β-globin gene transfected into 3T6 cells. The sequence homology between these upstream regions from all genes studied however is not as strong as that found for the TATA box. This suggests that the eukaryotic promoter may be polymorphic in structure containing "class" or "gene" specific active regions. This type of sequence has been identified for genes regulated by the hormone progesterone in the chicken oviduct. Mulvihill *et al* (1982) have found a DNA sequence in the progesterone responsive genes 250 to 300 nucleotides upstream of the 5' cap site which preferentially binds the hormone:receptor complex. This sequence may constitute another part of the promoter element for this class of hormone responsive genes.

The existence of more complex regulatory mechanisms is suggested by the discovery of Young *et al* (1980). The mouse α-amylase gene was shown to be expressed in a tissue specific manner utilizing two different mRNA start sites. This tissue specific expression is due to the use of separate promoters which function with different initiation efficiencies (Schibler *et al*, 1983). Benyajati *et al* (1983) have also shown that in *Drosophila melanogaster* the developmental stage-specific expression of the alcohol dehydrogenase (ADH) gene is brought about by using distinct promoter sequences which are reflected in the sequences of the mature mRNA at each stage.

Robins *et al* (1982) have shown that transcription of a cloned human growth hormone gene transfected into mouse L cells is responsive to the induction by glucocorticoids.

However, the mouse L cell growth hormone gene is refractory to any such hormone induction. These examples all suggest that other mechanisms are acting to regulate the gene's ability to be transcribed and thus the effectiveness of the promoter region in this initiation. It has been suggested that one of the requirements for gene expression is a chromatin structure which allows active transcription (Weisbrod, 1982). Thus before gene transcription can occur the promoter must be potentiated for expression.

CHROMATIN STRUCTURE AND GENE EXPRESSION

The DNA in eukaryotic cells is found in the form of chromatin, a highly specialized matrix of proteins and DNA. The basic subunits of this structure are the nucleosomes which are found in a linear repeating array along the DNA molecule. The higher order organization arises upon interaction of these nucleosomes with each other and numerous non-histone proteins.

Weintraub and Groudine (1976), first provided evidence that an altered chromatin structure was correlated with stage specific expression of the chick globin genes. This altered chromatin structure was manifested in a preferential sensitivity of the gene being expressed to the endonuclease DNase I. This sensitivity to DNase I was in part due to the association of the non-histone proteins HMG 14 and HMG 17 (Weisbrod and Weintraub, 1979; Weisbrod *et al*, 1980) with the chromatin. DNase I sensitivity is a feature of a number of actively transcribed genes, including the ovalbumin gene (Garel and Axel, 1976), the conalbumin gene (Kuo *et al*, 1979), and the *Drosophila* heat shock genes (Wu *et al*, 1979), and is a general phenomenon of all actively expressed genes producing nuclear RNA in tissue culture cells (Weisbrod *et al*, 1980).

When the DNase I digestion of active genes is reduced to limit the extent of enzymatic digestion, specific sites, extremely sensitive to this type of treatment, are manifested. These hypersensitive sites are found in a 200-300 base pair region of DNA at both the 5' and 3' ends of most actively transcribed genes (for review see Elgin, 1981). Their appearance can preceed overt expression and thus are thought to be required for subsequent gene expression and not just a reflection of it.

A correlation between the occurence of DNase I hypersensitive sites and gene activity was shown by Shermoen and Beckendorf, (1982) for the Sgs-4 gene in *Drosophila* which codes for a glue protein expressed only during pupariation. Muskavitch and Hogness (1982) studying variants unable to express this protein, have shown that this inability correlates with the deletion of one or more of the DNase I hypersensitive sites. McGinnis *et al* (1983) have also shown that a single base pair change within a hypersensitive region correlates with a reduction in Sgs-4 expression.

Recently, Burch and Weintraub (1983) have shown that the chicken vitellogenin gene manifests a hormone dependent DNase I hypersensitive site only in the liver, where gene expression takes place. Thus it seems that the DNase I hypersensitive sites reflect an altered conformation of chromatin which allows this enzyme to access and digest the underlying DNA in specific places.

Larsen and Wientraub, (1982) have shown that DNase I hypersensitive sites may have an altered DNA structure as evidenced by their extreme sensitivity to digestion by single strand specific nucleases like S1. Weintraub (1983) has shown that these specific S1 sensitive sites are evident in supercoiled, but not in nicked-chimeric plasmids, suggesting that tortional stress on the DNA backbone induces their formation. The author also shows that a chimeric plasmid's specific S1 sites are maintained in chromatin after transfection and that these regions seem to have a substantially lower affinity for nucleosomes. This lack of nucleosomes may be enough to exert a localized tortional stress producing S1 sites in the native chromatin.

What are these DNase I hypersensitive and S1 sensitive sites a reflection of? Interestingly, the transcription enhancer element found near the origin of replication in the SV-40 genome, has been shown to contain DNase I hypersensitive sites (Saragosti *et al*, 1982) and is devoid of nucleosome structures (Varsharsky *et al*, 1979; and Saragosti *et al*, 1982). Nordheim and Rich (1983) have recently shown that the negative supercoiling of the SV-40 virus DNA produces three specific regions where the transition from B to Z-DNA occurs. These regions are found specifically inside and adjacent to the enhancer element and are closely related in location to the DNase I hypersensitive sites in this region. The sites which form Z-DNA were found to be alternating purine:pyrimidine tracts about 8 base pairs in length and seem to occur in the activator or regulatory regions of all virus systems examined (Nordheim and Rich, 1983). It has been shown that elimination of two of the three potential Z-DNA sites in the SV-40 enhancer results in the loss of enhancer activity when the modified element is attached to the globin gene (Banerji *et al*, 1981). Thus the correlation between DNase I hypersensitive sites, S1 sensitive sites and altered DNA structure with the enhancer element suggests that the chromatin induced S1 sensitive sites described by Weintraub (1983), may involve the formation of Z-DNA. It is possible that the DNase I hypersensitive sites found around most active genes are cellular forms of these viral enhancer sequences. Enhancer type sequences have now been identified in both mouse (Fried *et al*, 1983) and human genomes (Conrad and Botchan, 1982). Recently, a cellular enhancer sequence was found to stimulate immunoglobulin transcription after recombination rearrangement from within an intron at a position 3' to the mRNA start site (Gillies *et al*, 1983; Banerji *et al*, 1983; and Queen and Baltimore, 1983). Thus, the identification of enhancer type sequences involved in the regulation of gene expression is becoming more common.

Finally, correlations between the states of DNA methylation and gene expression have been observed. All eukaryotes studied, except *Drosophila* and nematodes, contain minor but detectable amounts of the modified base 5-methyl cytosine (see Razin and Riggs, 1980, and Doerfler, 1983 for reviews). The discovery by Waalwijk and Flavell (1978) that methylated cytosines could be discerned from their non-methylated counterparts within the DNA sequence CCGG led to the implication that all actively expressed genes contained fewer 5-methyl cytosine moieties than when these same genes were dormant. It is known that *in vitro* methylation of the cytosine residues of cloned genes adversely affects transcription by possibly modifying the interactions of proteins with the DNA (Vardimon *et al*, 1982). Recently Busslinger *et al* (1983) have shown that methylation of the 5' regions of the γ-globin gene prior to transfection into L cells dramatically affects gene expression in clones where this gene has been stably incorporated.

Thus it seems that the chromatin structure in and around a gene affects its ability to be expressed and this initial potentiation is necessary for subsequent expression.

POST-TRANSCRIPTIONAL CONTROLS OF GENE EXPRESSION

The initiation of transcription is only the beginning of a complex pathway to produce translatable mRNA from a eukaryotic gene. Most eukaryotic mRNAs studied have been shown to contain a modified nucleotide attached in a novel linkage to the 5' end of the transcript (Shatkin, 1976). This cap structure has been shown to be involved in the binding of the 40 S ribosome subunit to the mRNA and translation initiation (Kozak and Shatkin, 1977). Green *et al* (1983) have recently shown that this cap is necessary for mRNA stability, as uncapped mRNA injected into the nucleus of *Xenopus* oocytes is rapidly degraded. The only case where the cap structure has been shown to be involved in the regulation of gene expression is that described by Kastern *et al* (1982). These authors show that maternal mRNA found in the unfertilized oocyte of *Manduca sexta* contains an unmethylated guanosine cap structure which appears to preclude these mRNAs from translation. Upon fertilization the mRNA caps on the *Manduca* embryo transcripts were of the methylated variety. These authors suggest that this unmethylated cap structure may inhibit the interaction of these mRNAs with the translation machinery in the oocytes but upon fertilization cap methylation allows transcript expression.

Selection of transcription termination and polyadenylation sites can act to regulate gene expression in a complex transcription unit. Both these mechanisms have been shown to modulate the expression of the eukaryotic virus Adenovirus-2. The use of alternate transcription termination sites has been shown to allow specific genes to be utilized while others further down stream in the viral genome are not transcribed at certain times during virus infection. The selective use of polyadenylation sites in a multi-cistronic primary transcript determines the efficiency in the expression of specific portions of the genome (Nevins and Wilson, 1981; and Shaw and Ziff, 1980).

Transcription termination may also be used to control the developmental expression of a specific 3' exonic sequence coding for a membrane anchor peptide during the expression of the IgM μ-heavy chain gene (Early *et al*, 1980; Alt *et al*, 1980). The tissue specific expression of an additional 3' exon, found in the thymus but not in the hypothalmic transcript from the rat calcitonin gene, also seems to be due to controlled transcription termination (Amara *et al*, 1982).

Following transcript termination and polyadenylation, those mRNAs produced from genes whose coding sequences are interrupted with intervening sequences (introns) must have these elements precisely removed prior to their export from the nucleus. It has been proposed that the splicing mechanism involves the removal of introns in multiple excision:religation events, each progressively removing a portion of the remaining intron (Kinniburgh and Ross, 1979; and Avvedimento *et al*, 1980). The exact mechanism of intron removal is not yet understood, however the process seems to be remarkably versatile, possessing the ability to accurately splice foreign (Green *et al*, 1983) and chimeric (Chu and Sharp, 1981) genes transfected into heterologous *in vivo* cellular systems. The intron:exon splice junction sequences seem to be important in maintaining efficient splicing (Wieringa *et al*, 1983), however only the dinucleotides GT, at the 5' end, and AG, at the 3' end, of each intron are absolutely conserved (see Breathnach and Chambon, 1981 for review). Recently Langford *et al* (1983) have also shown that a foreign intervening sequence inserted in the yeast actin gene is not spliced from the hybrid transcript. Langford and Gallwitz (1983) have shown that this is due to a specific octanucleotide sequence found in the 3' end of the yeast actin gene. This sequence is absolutely required for the removal of this intron and is found within the introns of three other protein coding nuclear genes from yeast. The authors suggest that this yeast specific sequence may be involved in intron recognition and binding of the RNA splicing apparatus.

The role of splicing in control of gene expression has become a central issue. Alternative splicing plays an important role in genome expression for the DNA tumor viruses (see Ziff, 1980 for review). Selective splicing has also been found to be involved in the expression of the immunoglobulin delta gene (Maki *et al*, 1981) and the mouse αA crystallin gene (King and Piatigorsky, 1983). Thus the splicing pattern used can have major effects on gene expression, as in the DNA tumor viruses, and is also important in the expression of complex eukaryotic genes.

CYTOPLASMIC CONTROL OF GENE EXPRESSION

Evidence exists that cytoplamic messenger RNA stability and decay are regulated in a way that contributes to control of gene expression in eukaryotes. Akusjarvi and Persson, (1981) suggest that the abundance of some viral mRNA transcripts found during Adenovirus infection is regulated by differential stability. Guyette *et al* (1979) have shown that the half-life of the casein mRNA transcript from cultured mammary gland tissue increases a dramatic 20 fold when stimulated by the hormone prolactin. The production of vitellogenin and a Very Low Density Lipoprotein (VLDL) can be induced in roosters by the administration of estrogen. This hormone has been shown to stimulate transcription of these genes, but also substantially increases the half-lives of these transcripts in some way (Wiskocil *et al*, 1980). Also, during aggregation of single cells into a multicellular structure in the slime mold *Dictyostelium*, certain developmentally regulated genes are expressed as evidenced by the production of their mRNA transcripts. If this aggregation is interrupted and the cells dispersed, transcription of these genes is halted and the mRNA is specifically and rapidly degraded (Chung *et al*, 1981). Bastos *et al* (1977) have shown that during erythroblast differentiation in mammals many different mRNAs are produced whose half-lives vary, conserving the globin mRNA and degrading other mRNAs during the final stages of reticulocyte development. This type of mRNA stability may be modulated through specific interactions of the poly(A) tail with specific proteins in the mRNP complex (see Brawerman, 1981).

The final point at which control over gene expression may be exerted is transcript translation. The control of translation seen during the "heat shock" response has been studied in a number of systems. In *Drosophila* tissue culture cells the "heat shock" or "stress" response sharply curtails cellular protein synthesis of all but the so called "heat shock" proteins. This is reflected by a rapid disappearance of cellular polysomes (McKenzie *et al*, 1975). Recently, Ballinger and Pardue, (1983) have demonstrated that this reduced translation of all other mRNAs is due to a significant reduction in the efficiency of protein synthesis initiation and elongation on non-heat shock mRNA. Translational control may also be studied by following the recruitment of stored maternal RNAs onto the translation machinery after oocyte fertilization. This phenomenon has been studied in numerous systems (see Raff, 1980 for review). It has been proposed that these untranslated messenger RNA molecules are non-polyribosomal due to some general masking effect of the proteins found in the mRNP complex. Jenkins *et al* (1978) showed that mRNPs from sea urchin oocytes prove to be inefficiently translated in a cell free *in vitro* translation system. However, Moon *et al* (1982) have shown that mRNPs of greater purity did in fact translate efficiently under similar conditions. More recently Moon *et al* (1983), using sea urchin eggs and embryos, showed that the translation of the majority of mRNAs takes place in association with the cellular cytoskeletal framework of the developing embryo. Prior to fertilization there are substantially lower levels of polyribosomes associated with this structure, and this suggested to the authors that the association with cytoskeletal elements could be determining the translatability of these stored mRNAs.

Other mechanisms for controlling the translation of stored mRNAs have been inferred from studies in different experimental systems. In *Manduca*, as described earlier, all oocyte mRNAs lack the methyl residue found on the 5' cap of the mRNAs in fertilized embryos (Kastern *et al*, 1982). These types of studies have also been reported for the translational recruitment of specific gene RNAs in *Drosophila* (Mermod *et al*, 1980), in *Smittia* (Jäckle, 1980), and in the surf clam *Spisula* (Rosenthal *et al*, 1983). Jäckle (1980) has presented evidence that a dissociable component is involved in the translatability of maternal actin mRNA. Rosenthal *et al* (1983) have shown that an increase in the length of the 3' poly(A) tail is associated with the translation of some specific oocyte mRNAs. Thus gene-specific controls over mRNA transcript translatability can regulate gene expression in certain circumstances.

STUDYING CONTROLS OF ONTOGENY

Historically, studies into the process of development have focused on the physical or genetic interventions which alter or affect some aspect of the control of ontogeny. These so called "defect experiments" allow one to observe the process of ontogeny in a holistic manner. Alternatively, one may study the regulation of expression of particular genes throughout the process of ontogeny in an attempt to discover developmentally significant regulatory controls. The most useful types of genes for this type of analysis are those which are regulated throughout development in either a temporal or a spatially specific manner.

These types of analyses have been done using the chicken globin genes as a system to investigate the controls over gene expression (Weintraub *et al*, 1981). Recently, several other systems have been described in *Drosophila* which may be utilized to study the developmental regulation of specific genes. Alcohol dehydrogenase (ADH) is a developmentally regulated tissue specific enzyme (Ursprung *et al*, 1970). Benyajati *et al* (1983) have shown that at two specific stages of development the gene transcript is produced from different promoter elements with alternative splicing events. That is, the selection of a stage specific promoter and the proper splicing events are involved in the regulation of the developmental expression of this gene. Rozek and Davidson (1983) have shown that the *Drosophila* myosin heavy chain gene produces three stage specific transcripts from a single gene. That these transcripts differ from each other at their 3' ends is reflected by their size and sequence. This selective expression of different 3' exon sequences proves to be an interesting developmental phenomenon whose significance is still not understood.

Fyrberg *et al* (1983) have studied the expression of the six actin genes in *Drosophila melanogaster*. The expression of each is highly regulated in both a temporal and a tissue specific manner making this system another with promise for analysing developmentally significant regulatory signals.

Finally, another gene whose expression is regulated throughout ontogeny in *Drosophila* produces the enzyme dopa decarboxylase.

THE DOPA DECARBOXYLASE GENE:ENZYME SYSTEM

Dopa decarboxylase (DDC) is a key enzyme, converting L-dopa to dopamine, in the pathway for the production of the sclerotizing agent N-acetyl dopamine (Sekeris and Karlson, 1966). This agent is used to harden the newly formed insect cuticle. The appearance of DDC activity at stages requiring cuticle sclerotization has been studied in *Calliphora* (Shaaya and Sekeris, 1965), *Sarcophaga* (Chen and Hodgetts, 1974), *Aedes aegypti* (Schlaeger and Fuchs, 1974), and

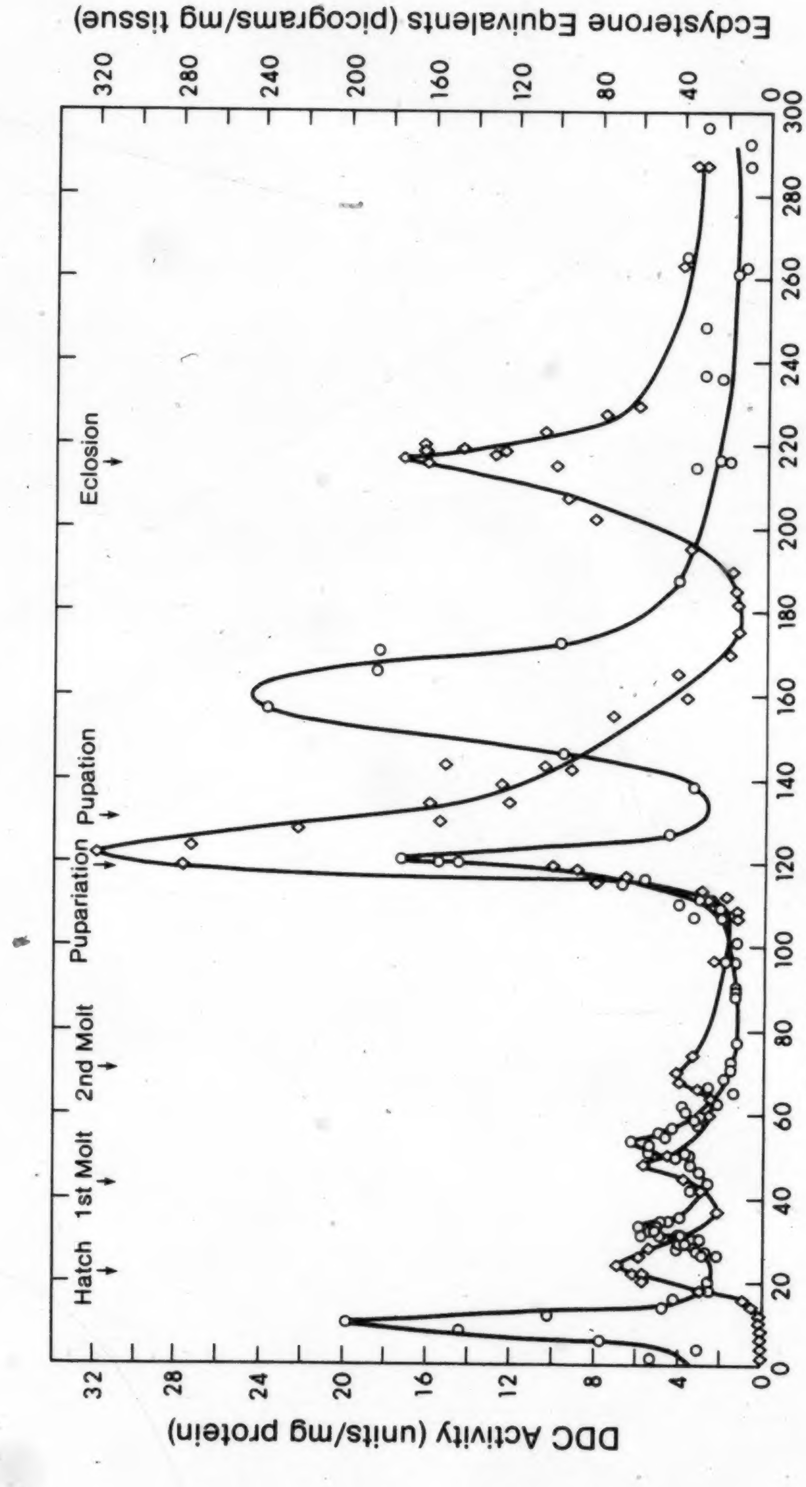
Drosophila melanogaster (Lunan and Mitchell, 1969).

In *Drosophila*, six peaks of DDC activity occur during insect ontogeny (Figure 1), all associated with stages that require cuticle sclerotization (Kraminsky *et al*, 1980; Marsh and Wright, 1980). Lunan and Mitchell (1969) first showed that the production of DDC activity is also tissue specific, as a large portion of the enzyme activity during pupariation is confined to the larval epidermis. Recently, Livingstone and Tempel (1983) have also shown, through genetic analysis, that the DDC gene product is involved in the production of dopamine in adult fly brains. This suggests that the DDC protein may also be specifically produced in the *Drosophila* nervous system. This tissue specificity and temporal modulation of DDC activity levels provides one with a model system for the study of factors regulating this gene's expression.

DDC EXPRESSION DURING PUPARIATION

Karlson and Sekeris (1966) showed in *Calliphora* that the appearance of DDC activity at pupariation was dependent upon the exposure of the epidermis to increased levels of the moulting hormone 20-OH ecdysone. Fragoulis and Sekeris (1975a, 1975b) demonstrated that this increase in DDC activity at pupariation represented *de novo* synthesis of the enzyme and could be correlated to the production of translatable DDC mRNA. In *Drosophila melanogaster* the rise in DDC activity and the increase in the ecdysteroid titer at

Figure 1: DDC activity and ecdysteroid titer profile throughout ontogeny in *Drosophila melanogaster*. This figure has been reprinted from Kraminsky *et al* (1980). The DDC activity and ecdysteroid titer measurements are represented by the open triangles and the open circles respectively. A unit of DDC activity in this figure is defined as: one nanomole of L-dopa decarboxylated/ 20 min assay/ mg protein.


Ecdysterone Equivalents (picograms/mg tissue)
320
280
240
200
160
120
80
40
0
DDC Activity (units/mg protein)
32
28
24
20
16
12
8
4
0
Hatch
1st Molt
2nd Molt
Pupariation
Pupation
Eclosion
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
Developmental Age (hour from egg deposition)

pupariation are concurrent (see Figure 1). In an elegant experiment utilizing the mutant *l(3)ecdysoneless-1*^{ts}, isolated by Garen *et al* (1977), Kraminsky *et al* (1980) showed that the appearance of DDC activity at pupariation also requires the rise in the hormone 20-OH ecdysone. Upon administration of dietary 20-OH ecdysone to third instar *l(3)ecdysoneless-1*^{ts} larvae developing at the restrictive temperature (29°C), accumulation of noticeable amounts of translatable DDC mRNA occurs within two hours. That this accumulation of DDC transcript still occurs in the presence of protein synthesis inhibitors (W.C.Clark, personal communication) suggests that this response is similar to the early puffing response of the salivary gland polytene chromosomes to 20-OH ecdysone stimulation described by Ashburner (1974). This early puff response is thought to be due to the interaction of the hormone:receptor complex with specific transcription signals on the DNA. The specific association of ecdysone:receptor complexes with chromosomal locations that produce puffs supports this hypothesis (Gronemeyer and Pongs, 1980).

At all other times during development in *Drosophila melanogaster*, the hormone and the DDC activity peaks occur at different times (Kraminsky *et al*, 1980; see Figure 1). This suggests that the relationship between the moulting hormone, 20-OH ecdysone, and the onset of DDC activity at these stages is different from that seen during pupariation. More evidence supporting this has recently been provided by Estelle (1983), who described a DDC activity variant in which the DDC expression at pupariation is lower than wild-type, while at all other times the DDC gene is expressed at a slightly higher rate. The phenotype of this variant also suggests that at pupariation the control of DDC expression differs qualitatively from that at other times when the gene is active. This temporal lag between the hormone peak and the onset of DDC activity at these other stages, may reflect an inter-relationship between them similar to that described for the so called "late puffing response" (Ashburner, 1974). Alternatively, some other reason may account for this temporal lag at stages other than during pupariation.

The primary objective of this thesis was to investigate the relationship between the embryonic ecdysteroid titer peak and the production of DDC activity just prior to the hatching of the first instar larvae. An attempt was made to find the reason for the temporal lag in DDC expression following the hormone DDC expression by investigating the following questions:

1) Are the ecdysteroids detected during embryogenesis of a different type than those at pupariation?

2) Is the DDC activity found during embryogenesis produced from a different structural gene than that at pupariation?

In response to the first question the embryonic ecdysteroids were analysed using TLC and HPLC methodology. The answer proved to be negative. In response to the second question the embryonic DDC molecule was compared to the puparial enzyme by both immunological and genetic criteria. The answer was again negative.

I then considered the possibility that the relationship between the hormone peak and the expression of the DDC gene was identical to that at pupariation but only appeared to differ due to the interaction of other factors causing a delay in the appearance of DDC activity. The question: does the ecdysteroid peak cause immediate accumulation of an untranslatable DDC gene transcript, was investigated. Northern gel blot analysis of the RNA transcript pools from samples collected throughout embryogenesis was carried out using a DDC clone kindly provided by Dr. J. Hirsh. This analysis showed that the production of DDC RNA transcripts did not occur until just prior to the onset of DDC activity. From these analyses it seems that the relationship between the hormone peak and DDC expression during embryogenesis differs from that seen a pupariation and is more similar to the late puffing response.

Other interesting observations were also noted from these Northern gel transcript analyses. A transcript species with homology to the probe was discovered in the RNA pools from oocytes and preblastoderm embryos, and DDC transcripts of various sizes were observed in both early and late embryonic RNA samples.

The final part of the thesis deals with the characterization of the DDC transcripts found at both early and late embryogenesis and during pupariation. The transcripts were characterized for length, homology to DDC gene intron probes, subcellular localization and association with cellular translation machinery. Also *in vitro* translation and specific immunoprecipitation was undertaken to further analyse the gene products during embryogenesis. A model is presented which attempts to explain the number and relationship between these DDC transcripts in terms of maturation from the primary gene transcript to a translatable form.

II. MATERIALS AND METHODS

Drosophila melanogaster STOCKS

All fly stocks were maintained on a synthetic medium in constant darkness (Nash and Bell, 1968). The strains, *l(3)ecdysoneless-1*^{ts} (Garen *et al*, 1979) and *l(2)Ddc*^{ts2} (Wright *et al*, 1981) were kept at 20°C. A strain isogenic for the second chromosome of Canton-S was used to represent wild type in all studies.

EMBRYO COLLECTIONS

All embryo collections were done in constant darkness at 20°C. Newly eclosing flies were released into a plexiglass cage and allowed to feed for a few days on trays of synthetic food liberally smeared with live yeast paste. Embryos were collected on trays of laying medium (1.2%(w/v) agar, 1.0%(w/v) sucrose) smeared with live yeast paste. Collections were 2 hr in duration, after which the trays were placed at the appropriate temperature for aging. Sample ages were timed from the midpoint of the collection period.

The embryos were harvested at the appropriate times by scraping the yeast paste off the tray and flooding it with distilled water. The eggs were gently freed from the medium using a fine hair brush. The embryos were then collected into Nitex mesh and washed extensively with distilled water. The embryos were dechorionated by washing with 2.5%(w/v) sodium hypochlorite for 90 sec, followed by 70%(v/v) ethanol, then distilled water, and frozen using liquid nitrogen. The samples were then stored at -40°C until needed.

OVARY DISSECTION

Canton-S virgin females were aged from 2 to 4 days at 25°C. Twenty-five flies were anaesthetized by cooling on ice and single flies were then dissected in a drop of insect ringer (0.75%(w/v) NaCl, 0.035%(w/v) KCl, 0.021%(w/v) $CaCl_2$, Ephrussi and Beadle, 1936). The dissected ovaries were transferred to one ml of D-20 tissue culture medium (Echalier and Ohanessian, 1970), generously supplied by Charlotte Spencer, in a 1.5ml microcentrifuge tube. The excess medium was drawn off and the ovaries were frozen using liquid nitrogen and stored at -40°C until needed.

ECDYSTEROID ANALYSIS SAMPLE PREPARATION

Radioimmunoassays for ecdysteroids were performed in the laboratory of Dr. J.D. O'Connor (U.C.L.A.) on extracts derived from live dechorionated embryo samples homogenized using a motor driven teflon pestle:glass tissue grinder (Bellco) at 0°C in 70% methanol, 1×10^{-3}M phenylthiourea at a tissue concentration of 75mg wet weight/ml. The homogenates were cleared by centrifugation at 12,000xg for 10 min at 4°C. The supernatants were stored at -40°C until needed. For identification and quantification of ecdysteroids by TLC and HPLC the samples were homogenized at 200mg tissue wet weight/ml.

DOPA DECARBOXYLASE ASSAYS

All assays for dopa decarboxylase activity were done as described in Clark *et al* (1978). One unit of activity corresponds to the decarboxylation of 1 nmole of L-dopa per hr under standard assay conditions.

PROTEIN DETERMINATIONS

The protein concentration in crude extracts was estimated using the procedure of Bradford (1976) or the procedure of Lowry *et al* (1951) using BSA as the standard.

IgG AFFINITY CHROMATOGRAPHY

The antibody used throughout this thesis was raised against puparial DDC purified to homogeneity in the laboratory of R.B. Hodgetts. The immuno-γ-globulin fraction of whole rabbit serum was purified as described by Goding (1979). The rabbit sera were diluted with an equal volume of PBS (20mM sodium phosphate (pH 7.4), 130mM NaCl), and applied to a 10ml protein A-Sepharose CL4B column (Pharmacia) equilibrated with PBS at a flow rate of 10ml/hr at 4°C. The column was washed extensively with PBS at 20ml/hr flow rate. Then the bound IgG was eluted with 150mM NaCl, 0.58%(v/v) acetic acid. The eluate was collected and dialysed overnight against one liter of PBS with one change. The dialysed fraction was then concentrated using an Amicon

Ultrafiltration cell Model 52 and a PM-10 membrane.

DOUBLE IMMUNODIFFUSION

Double immunodiffusion analysis was performed in a fresh IDF 1 cell (Cordis Laboratories) with extracts at a tissue concentration of 100mg wet weight/ml in 10mM sodium phosphate buffer (pH 7.2) containing 150mM NaCl, 2mM dithiothreitol, 0.13mM pyridoxal phosphate. Sample wells were loaded with 50µl and the center well was loaded with 15µl of monospecific anti-DDC antibody that had been labeled with ^{125}Iodine (2.88µg, 0.223µCi/µg) using the lactoperoxidase method (David and Reisfeld, 1974) by the Edmonton Radiopharmaceutical Center. The plate was developed for 24hr at 20°C. The agarose disc was then removed from the plate and incubated in a wash solution containing 200mM NaCl and 0.1%(v/v) Triton X-100 for 24hr at 20°C, then stained with Coomassie brillant blue R-250 (0.05% w/v), in 10%(v/v) acetic acid and 25%(v/v) isopropanol. Destaining was accomplished with 7%(v/v) acetic acid. The disc was then vacuum dried to 3MM Whatman chromatography paper and autoradiographed at -40°C.

RETICULOCYTE LYSATE PRODUCTION

Young male Dutch belted rabbits were used for reticulocyte lysate production. The rabbits were made anemic by subcutaneous injection of a neutralized 1%(w/v) aqueous solution of phenylhydrazine hydrochloride at a dose of 1 ml/Kg weight. They were injected for 5 consecutive days. The blood was collected via heart puncture on the 7th day of the schedule. The blood was heparinized during collection to prevent clotting. The red cells were then washed 4 times with saline (130mM NaCl, 5mM KCl, 7.5mM $MgCl_2$) and collected by centrifugation at 480xg for 2 min.

The cells were lysed by the addition of 1.5 volumes of ice cold distilled deionized water and cellular debris was pelleted by centrifugation at 30,000xg for 15 min at 4°C. The supernatant was aliquoted and frozen with liquid nitrogen then stored until needed at -70°C.

Messenger RNA dependant lysate was produced exactly as stated in Pelham and Jackson (1976). While thawing the lysate it was made 50μg/ml for creatine kinase and 25μM for haemin using stock solutions of 5mg/ml creatine kinase in 50%(v/v) glycerol and 1mM haemin in 90%(v/v) ethylene glycol, 20mM Tris-HCl (pH 8.2 at 30°C), 50mM KCl. To digest endogenous mRNA this lysate was brought to 1mM $CaCl_2$ and 10μg/ml micrococcal nuclease using stock solutions of 0.1M $CaCl_2$ and 1mg/ml nuclease (dissolved in distilled water and stored at -40°C). The mixture was then incubated for 7 to 10 min at room temperature for digestion, then made 2mM EGTA from a 0.1M stock which had been neutralized with KOH. This lysate was then treated with fresh aqueous diisopropylfluorophosphate to a final concentration of 1.6mM to reduce endogenous proteolytic activity. This treatment had no apparent effect on lysate translatability. The lysate was then divided into small aliquots, frozen using liquid nitrogen, and stored at -70°C.

RNA EXTRACTION

RNA was extracted following the method of Spradling *et al* (1975) with some modification. All glassware and pipettes were dry heat treated at 170°C for at least three hours before use. The samples were homogenized on ice in RNA extraction buffer using a motor driven teflon pestle in a Wheaton glass homogenizer at a tissue concentration of 1:20 (w/v) or less. The homogenates were made in 30mM Tris-HCl (final pH 7.5 at 4°C) containing 100mM NaCl, 5mM KCl, 10mM $MgSO_4$, 5mM dithiothreitol, 4mM EGTA, 5mM NEM, 0.5%(v/v) 2-mercaptoethanol, 0.5%(v/v) Nonidet P-40, 25μg/ml polyvinyl sulfate, 35μg/ml spermine. Sodium dodecyl sulfate was added after homogenization to a final concentration of 1%(w/v). The homogenate was extracted twice with buffer-saturated phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). If necessary the first phenol interphase was re-extracted with additional RNA extraction buffer. The RNA was precipitated from the final aqueous phase by the addition of 2.5 volumes of 95%(v/v) ethanol and storage at -40°C. This yielded a mixture of nuclear and cytoplasmic RNA. If cytoplasmic RNA was desired the initial extract was cleared of the nuclei and cellular debris by centrifugation at 12,000xg for 10 min. The supernatant was then extracted as above after the addition of SDS.

OLIGO-dT CHROMATOGRAPHY

The oligo deoxythymidilate chromatography was as described by Aviv and Leder (1972) with minor modification. The oligo-dT cellulose (type 7, P.L. Biochemicals) was equilibrated in application buffer (10mM Tris-HCl (pH 7.5 at 22°C), 400mM NaCl, 0.5%(w/v) SDS). A small column was poured into a heat treated pasteur pipette, packed and equilibrated at 10ml/hr using application buffer. The RNA to be chromatographed was dissolved in elution buffer (10mM Tris-HCl (pH 7.5 at 22°C), 0.05%(w/v) SDS) followed by the addition of more NaCl and SDS to make application buffer. After the RNA was applied, the column was washed with application buffer. When the absorbance (254nm) of the wash reached baseline, the poly(A) mRNA was washed from the column with elution buffer. The fraction containing poly(A) RNA was collected and precipitated at -40°C by the addition of sodium acetate to a final concentration of 200mM and 2.5 volumes of 95%(v/v) ethanol.

NORTHERN ANALYSIS OF RNA

RNA Electrophoresis

RNA samples were electrophoresed on formaldehyde agarose gels as described by Chung *et al* (1981) with some modification. All operations with these gels were carried out in a fume hood. The resolution of the original horizontal-submarine gel system was improved by utilization of a combined horizontal:vertical (H:V) gel system. A solution of 1.35% to 1.5%(w/v) agarose in RNA electrophoresis buffer(20mM Sodium borate pH8.3, 0.1mM EDTA, or 20mM EEPS pH8.0, 10mM Na acetate, 0.1mM EDTA) was melted and cooled to 60°C and formaldehyde was added to 10%(v/v) from a 37%(w/w) stock solution. A 3mm thick gel was then cast, in a horizontal position, between one notched and one frosted glass plate sealed with masking tape completely around the perimeter. The slot comb was inserted perpendicularly to make sample slots for horizontal phase electrophoresis.

The RNA samples to be electrophoresed were precipitated then dissolved in distilled deionized water at a concentration of 1mg/ml. A 2μl aliquot of each RNA sample was added to a denaturation solution of 50%(v/v) formamide, 30%(v/v) stock formaldehyde 37%(w/w), 20mM sodium borate pH 8.3, 0.1mM EDTA to give a final volume of 20μl, then heated at 65°C for 2 to 5 min. After cooling 2μl of 50%(v/v) glycerol containing 0.15%(w/v) BPB and 0.15%(w/v) xylene cyanol was added to each sample. The masking tape was then removed from sample slot area and its opposite edge to allow contact with the electrophoresis wicks. The gel was placed in a horizontal electrophoresis apparatus and 3MM Whatman wicks soaked in RNA electrophoresis buffer plus formaldehyde (10%v/v) were positioned to ensure good contact. The samples were loaded and electrophoresed toward the anode at 90mA constant current for about 30 min. The gel was then transferred out of the horizontal unit into a vertical apparatus. This mode of electrophoresis allows samples to migrate more efficiently with lower gel temperatures. Once the transfer was complete the electrophoresis was continued toward the anode at a constant current of 45 mA. The buffer, during these runs (usually 17 to 20 hr), was constantly recirculated between the upper and lower tanks at 200 ml/hr. This avoided ion depletion which initially was a problem in runs of this length.

After the run was completed the slots containing rRNA size markers were cut from the gel and stained in a fresh solution of 200mM ammonium acetate containing 0.5µg/ml ethidium bromide for approximately 2 hr with gentle shaking. The gel slices were then washed for 30 min in 200mM ammonium acetate and the RNA visualized using a long wave UV light transilluminator (Ultra-violet Products Inc, San Gabriel, CA).

Northern Blotting

RNA was transferred from agarose formaldehyde gels to nitrocellulose paper by blotting in a high salt solution (Thomas, 1980). The formaldehyde gel was placed on a wick of 3MM Whatman paper saturated in 10xSSC (1.5M NaCl, 0.15M sodium citrate) that extended into a reservoir of the same. The gel was overlaid with a sheet of nitrocellulose that had been wetted with water and then equilibrated with 10xSSC. The nitrocellulose was overlaid with 10 sheets of 3MM Whatman soaked in 10xSSC, and then a weighted stack of dry paper towels. The gel was blotted for 12 to 18 hr. The nitrocellulose was removed from the gel, the positions of the rRNA standards marked, and the blot was then baked for a minimum of 2 hr in a vacuum oven at 80°C.

Hybridizations

Nick-translated DNA probes were hybridized to RNA immobilized on nitrocellulose as described by Thomas (1980). The baked nitrocellulose filters were prehybridized at 42°C for a minimum of 4 hr in 5 to 20ml of a solution containing 50%(v/v) formamide, 5xSSC, 40mM sodium phosphate buffer (pH 6.5), sonicated and denatured salmon or herring sperm DNA at 250µg/ml, and 0.02%(w/v) each of BSA, Ficoll and polyvinylpyrrolidone (1x Denhardt's).

The hybridization took place in 10 to 20ml of the same buffer or 4 parts buffer plus 1 part 50%(w/v) dextran sulfate (M.W. 5 x 10^5, Sigma). The nick-translated probes were denatured by dilution into hybridization buffer minus dextran sulfate and incubation in a boiling water bath for 2 min. The probe was cooled to the hybridization temperature and added to the hybridization bag. Twenty to sixty million cpm of fresh radioactive probe (sp.act. approx. 3×10^8cpm/μg) was used for every blot hybridization. The hybridizations were carried out at 42°C in a water bath with gentle shaking for 12 to 48 hr.

The blots were washed twice in 250ml of a solution of 2xSSC, 0.1%(w/v) SDS for 5 min each at room temperature, and twice in 0.1xSSC, 0.1%(w/v) SDS for 15 min each at 50°C. The moist blots were wrapped in Saran wrap prior to autoradiography.

Autoradiography

Hybridized RNA blots were exposed to Kodak XAR-5 film in X-ray cassettes (Picker) mounted with Cronex intensifying screens (Dupont) at -70°C. After sufficient exposure the film was developed manually using Kodak X-ray developer and Kodak Rapid Fixer or suitable equivalent following the manufacturer's instructions.

SUCROSE GRADIENT RNA FRACTIONATION

Poly(A) RNA was size fractionated on sucrose gradients following the procedure of Anderson and Lengyel (1979) with some modification. Linear 15%-30%(w/v) sucrose gradients were poured into nitrocellulose SW-40 rotor tubes (Beckman). The sucrose solutions were made in 5mM Tris-HCl (pH7.5 at 22°C) containing 100mM NaCl, 1mM EDTA, using a 10x stock solution which had been treated with DEPC to 0.1%(v/v) and autoclaved. After sucrose dissolution, SDS was then added to a final concentration of 0.5%(w/v) using a 10%(w/v) stock solution. The gradients were equilibrated at room temperature for 1 hr to allow settling of discontinuities.

The RNA to be fractionated was precipitated and dissolved in 5mM Tris-HCl (pH7.5) containing 1mM EDTA and 0.5%(w/v) SDS. The RNA was heated to 60°C for 1 min, quickly cooled to room temperature and layered onto the gradients. The gradients were centrifuged at 40,000 rpm for 7.5 hr at 22°C.

Each gradient was fractionated by bottom displacement into 0.4ml aliquots using an ISCO Density Gradient Fractionator Model 640. The RNA from each fraction was then precipitated overnight at -40°C by the addition of 40µl of a solution containing 1.25M sodium acetate and 1.25M ammonium acetate and 1.1ml of 95%(v/v) ethanol. The RNA was collected by centrifugation at 12,000xg for 60 min at 0°C. The supernatant was aspirated and the pellet dissolved in 300µl of distilled deionized water. The RNA was precipitated again by adding 30µl of 2.0M Na acetate and 850µl of 95%(v/v) ethanol.

RNA DOT BLOTS

RNA samples from sucrose gradient fractions were dot blotted on nitrocellulose equilibrated in 20xSSC using a Tyler Research dot blot apparatus following the procedure described by Thomas (1980). Fifty µl of 20xSSC was dispensed into each sample well and up to 2µg of RNA was added. A vacuum was applied for 5 min to draw the liquid through the nitrocellulose membrane. The paper was then baked for 2 hr at 80°C in a vacuum oven. The baked dot blots were hybridized following the procedure used for northern gel blots.

In Vitro TRANSLATION

A typical translation reaction contained 60%(v/v) messenger dependant lysate, 10mM HEPES-KOH (pH7.7), 100mM potassium chloride, 0.5mM magnesium acetate, 10mM creatine phosphate, 25uM of each amino acid (excluding methionine), 250µCi/ml ^{35}S-methionine (1000-1300 Ci/mmole, 5mCi/ml), and poly(A) mRNA at 25µg/ml. All reactions were carried out at 37°C. Protein synthesis was terminated by the addition of 1 volume of NETE buffer (50mMTris-HCl (pH 7.5 at 4°C), 150mM NaCl, 5mM EDTA, 5mM EGTA) plus 0.5%(w/v) Triton X-100.

Incorporation of ^{35}S-methionine into translation products was determined using the procedure of Mans and Novelli (1961). Samples of the lysate were spotted onto squares (1cm) of Whatman 3MM paper, and placed in 5%(w/v) TCA in a boiling water bath for 10 min. The samples were then rinsed in cold 5%(w/v) TCA for 10 min, in 95%(v/v) ethanol for 2 min, in ether for 2 min, and then allowed to air dry. The filters were then immersed in 5 ml of Aquasol-2 (New England Nuclear) and the radioactivity determined in a Beckman LS-7500 liquid scintillation spectrometer.

IMMUNOPRECIPITATIONS

Translation samples to be immunoprecipitated were first incubated with various amounts of heterogeneous rabbit preimmune IgG on ice. This was done to reduce the amount of non-specific binding to the anti-DDC IgG. This preimmune IgG was removed using fresh *Staphylococcus aureus* ghosts, prepared as described by Kessler (1976). The resulting supernatant was then incubated with the anti-DDC monospecific antibody IgG for up to 12 hr on ice. The antigen:antibody complexes were removed by mild centrifugation following incubation with *S. aureus* ghosts for 30 min on ice. The pellet was washed three times using NETE + 0.05%(v/v) NP-40 and finally resuspended in SDS reducing buffer then heated to 65°C for 5 to 10 min. The cellular debris was pelleted by centrifugation at 12,000xg for 10 min and the supernatant was subsequently analysed using SDS-PAGE.

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Radioactive protein samples were analysed on SDS polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970) with the modifications of Kikuchi and King (1975).

FLUOROGRAPHY

SDS slab gels were prepared for fluorography using the method of Chamberlain (1979). Gels to be fluorographed were fixed in 10%(v/v) acetic acid for 1 hr, then washed with a minimum of 20 volumes of distilled water for 30 min to reduce acidity. The gel was finally soaked in 1.0M sodium salicylate for 30 min with moderate shaking and then dried onto Whatman 3MM paper using a vacuum gel drier. These gels were then exposed to X-Ray film (XRP-1, XAR-5) at -70°C.

HYBRID-ARRESTED TRANSLATION

A cloned DNA fragment containing DDC structural gene sequences kindly provided by Dr. J. Hirsh (Hirsh and Davidson, 1981) was tested for DDC mRNA homology using the methods of Evans and Rosenfeld (1979) with modification. The DNA samples to be tested were first denatured in 20mM HEPES-KOH (pH 7.3) containing 300mM KCl by incubation in a boiling water bath for 2 min. Four micrograms of mRNA from fraction 22 (Figure 10) was added to give a final volume of

20μl, then incubated at 70°C for 50 min. After the incubation the samples were frozen with liquid nitrogen, thawed, and 55μl of a potassium adjusted translation mixture were added. This was done to give a final potassium concentration of approximately 100 mM. This was incubated at 37°C for 90 min, then diluted with 1 volume of NETE plus 0.5%(v/v) Triton X-100, and immunoprecipitated. The resultant samples were analysed by SDS polyacrylamide gel electrophoresis.

POLYSOME ISOLATION

Polysomal analysis of embryo samples was carried out according to the methods of McKenzie *et al* (1975) with minor modification. Live embryo samples were homogenized by hand in a teflon pestle:glass tissue grinder (Bellco) at a tissue concentration of 150mg/ml. The polysome extraction buffer consisted of 50mM HEPES-KOH (pH 7.4) containing 250mM KCl, 10mM $MgCl_2$, 25mM EGTA, 0.5mg/ml heparin, 0.1%(v/v) diethylpyrocarbonate, 1%(v/v) Nonidet P-40, 0.25M sucrose, and 0.1 volume of rat liver supernatant prepared as described by Blobel and Potter (1966). The extract was cleared of cellular debris and nuclei by centrifugation at 12,000xg for 10 min at 4°C. The supernatant was then loaded onto a 13ml 0.5M to 1.5M linear sucrose gradient made in the same buffer minus NP-40. The gradients were centrifuged at 38,000 rpm for 4 hr at 4°C using a SW-40 rotor (Beckman).

The gradients were fractionated by bottom displacement using an ISCO Density Gradient Fractionator Model 640. The absorbance profile was also monitored using an ISCO UA-2 Ultraviolet Analyzer at 254nm. The resultant fractions were diluted with 1 volume of RNA extraction buffer then made 1%(w/v) SDS and frozen at -40°C until phenol extractions could be done. The poly(A) mRNA was isolated from each fraction as previously described.

BACTERIAL GROWTH MEDIUM

The medium used to grow all strains harbouring plasmids was Luria broth plus thymine (LT). This contained per liter: 12.0g Bacto Tryptone, 10.0g NaCl, 5.0g Bacto Yeast extract, 1.0g dextrose, and 50mg thymine. Solid complete was LT containing 12.0g Bacto Agar. All bacterial stains (HB101) used for plasmid preparation were grown in LT broth containing 100mg/L ampicillin for pBR322 derived plasmids, or 125mg/L spectinomycin for pACYC184 derived plasmids. These antibiotics were added after the medium was autoclaved and cooled to at least 50°C.

PLASMID ISOLATION

Bacterial plasmid DNA was purified on a cesium chloride: ethidium bromide density gradient following the method of Thompson *et al* (1974) with modifications. A 500 ml saturated culture was harvested and lysed as described with the lysozyme treatment increased to 5 min.

The cleared lysate was made to 1.25M NaCl by the addition of 1 volume of a 2.5M stock, and incubated on ice for 1 hr. The lysate was spun at 30,000xg for 10 min to pellet the high molecular weight DNA. The supernatant was collected and the DNA was precipitated by the addition of 0.54 volumes of isopropanol and incubation at -70°C for 1 hr. The precipitate was collected by centrifugation at 12,000xg for 10 min. The precipitate was dissolved in 7ml of TE using a rotary shaker for 1 hr at room temperature. The undissolved material was pelleted by centrifugation at 12,000xg for 10 min. One gram of CsCl was then dissolved for every ml of supernatant and finally 0.8ml of an ethidium bromide stock solution (10mg/ml) was added for every 10ml of supernatant. The samples were loaded into a VTi65 rotor (Beckman) and centrifuged at 50,000 rpm for a minimum of 6 hr.

The banded plasmid DNA was visualized using a long wave UV transilluminator. A syringe was used to puncture the tube and withdraw the plasmid DNA. The ethidium bromide was partitioned from the DNA by mixing the aqueous solution with an equal volume of water-saturated n-butanol. This was repeated until no ethidium in the aqueous phase was apparent. The DNA samples were finally dialysed overnight in 1L of TE with one change at 4°C.

During the late stages of this work the procedure of Holmes and Quigley (1981) was used to prepare the sample prior to CsCl centrifugation. The bacterial pellet from 500ml of culture was washed with 100ml of STE buffer (0.1M

NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA) via centrifugation at 4000xg for 10 min then resuspended in 10ml of the same buffer. Approximately 10-20mg of lyophilized lysozyme was dissolved and the mixture was incubated at room temperature for 5 min. This solution, in a 50ml Erlenmeyer flask, was heated over a bunsen burner until the liquid just started to boil then immediately transferred to a boiling water bath for a duration of 40 sec. The flask was then cooled in an ice:water bath for 5 min. The contents were finally centrifuged in a SW40 rotor at 25,000 rpm for 30 min at 4°C. The resulting supernatant was prepared for density gradient centrifugation as described above.

DNA RESTRICTION

Specific DNA fragments were excised from plasmid vector DNA using sequence specific restriction enzyme cleavage (Cohen *et al*, 1973). Reactions were carried out following the restriction enzyme manufacturer's specifications. Restrictions were incubated at 37°C for 2 to 4 hr after which 5µl of a dye solution (50%(v/v) glycerol, 0.15%(w/v) BPB and 0.15%(w/v) xylene cyanol) was added. The samples were either electrophoresed immediately or frozen at -40°C for later use.

DNA AGAROSE GEL ELECTROPHORESIS

Restriction enzyme digests were analysed by agarose gel electrophoresis (Cohen *et al*, 1973; Thomas and Davis, 1975). A solution of 1%(w/v) agarose (Sigma type II, medium EEO) in TAE (50mM Tris-acetate pH 8.3, 2mM EDTA$(Na)_4$) was melted and cast in a horizontal slab gel electrophoresis apparatus (Tyler Research Corp). Analytical gels were cast with 13 slots; preparative gels with a comb containing 2 large slots and 1 reference slot. The DNA was visualized on a long wave UV transilluminator after staining the gels with a solution containing 0.5μg/ml ethidium bromide for 30 min. Photographs were taken using a Polaroid MP4 camera with Polaroid type 57 film and a Kodak Wratten #9 gelatin filter.

NICK TRANSLATION

Radioactivity was incorporated into purified DNA as described by Davis *et al* (1977) with some modifications. Reactions of 50μl final volume contained 1μg DNA, 50mM Tris-HCl (pH 7.2), 10mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA (nuclease free), 0.02mM dATP, dGTP TTP, 0.107μM ^{32}P-dCTP (3000 Ci/mmole, 10 mCi/ml in Tricine, New England Nuclear), 10 units of E.coli DNA polymerase I and 0.25ng activated calf thymus DNase I. The reaction was carried out at 14°C for 3 hr then stopped by the addition of 1 volume of stop solution (20mM EDTA, 2mg/ml sonicated calf thymus DNA and 0.2%(w/v) SDS). A 2μl sample of a 0.15%(w/v) solution of BPB was then added to the reaction. This was loaded onto a 2ml

Sephadex G-50 pasteur pipette column equilibrated with TE. The column eluate was collected in 1 ml fractions until the BPB was evident. Specific activity of the radioactive DNA was determined by counting an aliquot of each fraction in 5ml of distilled water in a Beckman LS7500 liquid scintillation spectrophotometer.

DNA RESTRICTION FRAGMENT ISOLATION

DNA restriction fragments were purified from agarose gels by electroelution or phenol extraction.

Restriction fragments to be purified by electroelution were electrophoresed in a 1%(w/v) agarose gel with preparative slots as described earlier. The specific fragment was cut from the gel with a minimum of agarose. The slice was then cut into pieces and dispensed into the bottom half of a silanized 10ml glass pipette, which had been outfitted with a silanized glass wool plug. A dialysis bag was then attached to the bottom end of the pipette and clipped securely. Care was taken to insure no air remained trapped in the pipette between the agarose and the dialysis bag. The agarose was then stopped in the pipette with another silanized glass wool plug. The pipette was submerged in a horizontal electrophoresis apparatus filled with TAE buffer and electrophoresed with the dialysis bag to the anode using a constant current of 50mA overnight. The sample was then retrieved from the dialysis membrane in a minimum of buffer and precipitated by adding sodium acetate to 200mM and 2.5 volumes of 95%(v/v) ethanol.

The method of Weislander (1979) was used to recover DNA restriction fragments from low gelling temperature agarose gels. A solution of 1%(w/v) low gelling temperature agarose(Sigma, type VII) in TAE buffer was melted and cast into a horizontal slab gel electrophoresis apparatus using a preparative slot comb. The gel was allowed to set at 4°C. The restricted DNA samples were electrophoresed at room temperature until the desired band was resolved. The restriction fragment was then cut from the gel with a minimum of agarose. The agarose gel slice was put into a silanized Corex glass centrifuge tube with 5 to 10ml of TE buffer and then heated to 65°C until the agarose had completely melted. This was extracted twice with 1 volume of water-saturated phenol and twice with 1 volume of chloroform:isoamyl alcohol (24:1). The volume of the final aqueous phase was reduced, if necessary, by mixing with an equal volume of secondary butanol until the DNA could be finally precipitated as previously described.

III. RESULTS

DDC ACTIVITY AND ECDYSTEROID TITER DURING EMBRYOGENESIS

To begin the investigation both the DDC activity and ecdysteroid titer were ascertained during embryogenesis in *Drosophila melanogaster*. All ecdysteroid RIA analysis was kindly done by Dr. J. D. O'Connor at U.C.L.A. Figure 2 presents the data for the wild-type strain *Canton-S* maintained at 25°C. The ecdysteroid titer peaks at 10 hr of development. DDC activity on the other hand reaches a maximum just prior to, or during the first larval hatch, which occurs approximately 22 hr after egg deposition. Thus this demonstrates that there is a temporal lag between the ecdysteroid titer and DDC activity peaks of approximately 12 hr at this temperature.

THE EFFECT OF THE *l(3)ecdysoneless-1*^{ts} MUTATION DURING EMBYROGENESIS

The temperature sensitive lethal mutation *l(3)ecdysoneless-1*^{ts} was shown to eliminate the puparial peak of 20-OH ecdysone when mutant larvae are raised at the restrictive temperature of 29°C (Garen *et al*, 1977). Kraminsky *et al* (1980) used this mutant to demonstrate that the rise in DDC activity during pupariation required a prior increase in 20-OH ecdysone titer. Since I was interested in the relationship between ecdysteroids and the expression of DDC activity during embryogenesis the effect of the *l(3)ecdysoneless-1*^{ts} mutation at this stage was

Figure 2: Survey of DDC activity and ecdysteroid titer during embyrogenesis at 25°C.

Canton-S wild-type embryos were collected and allowed to develop at 25°C, then assayed for DDC activity using the method of Clark *et al* (1978). The graph values for DDC activity represent the average of two separate determinations. The ecdysteroid titer determinations were carried out on samples prepared as described in the Materials and Methods by Dr. J.D. O'Connor (U.C.L.A.). The time of the first instar larval hatch is indicated by the downward arrow in the upper right corner.

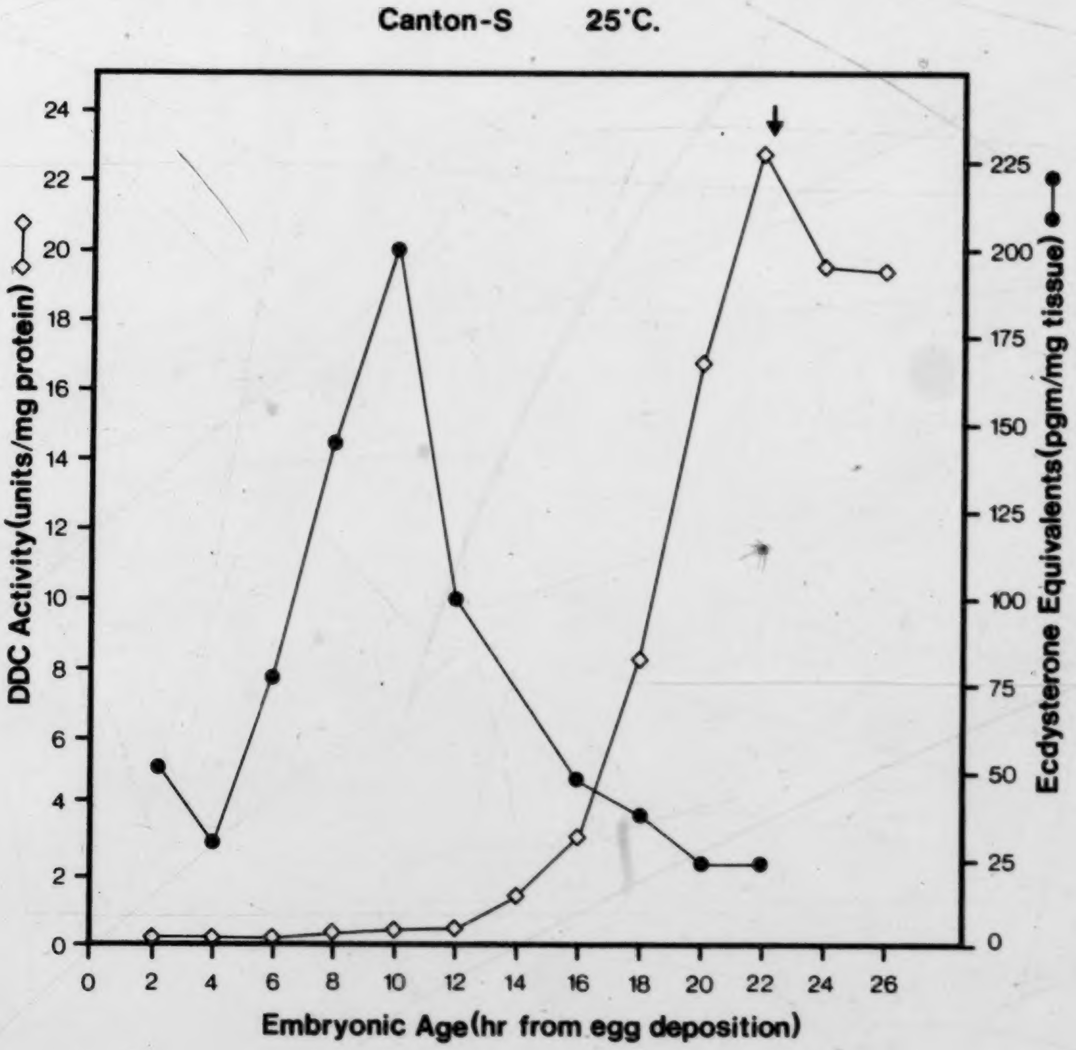

Canton-S 25°C.
DDC Activity(units/mg protein) ◇
Ecdysterone Equivalents(pgm/mg tissue) ●
Embryonic Age(hr from egg deposition)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
25
50
75
100
125
150
175
200
225

Figure 3: *Canton-S* and *l(3)ecdysoneless-1*'' DDC activity and ecdysteroid titer measurements during embryogenesis at 20°C.

Embyros from both *Canton-S* and *l(3)ecdysoneless-1*'' strains were collected and allowed to develop at 20°C and then assayed for DDC activity or ecdysteroid titer as in Figure 2. The time of the first instar larval hatch is indicated by the downward arrow in the upper right corner.

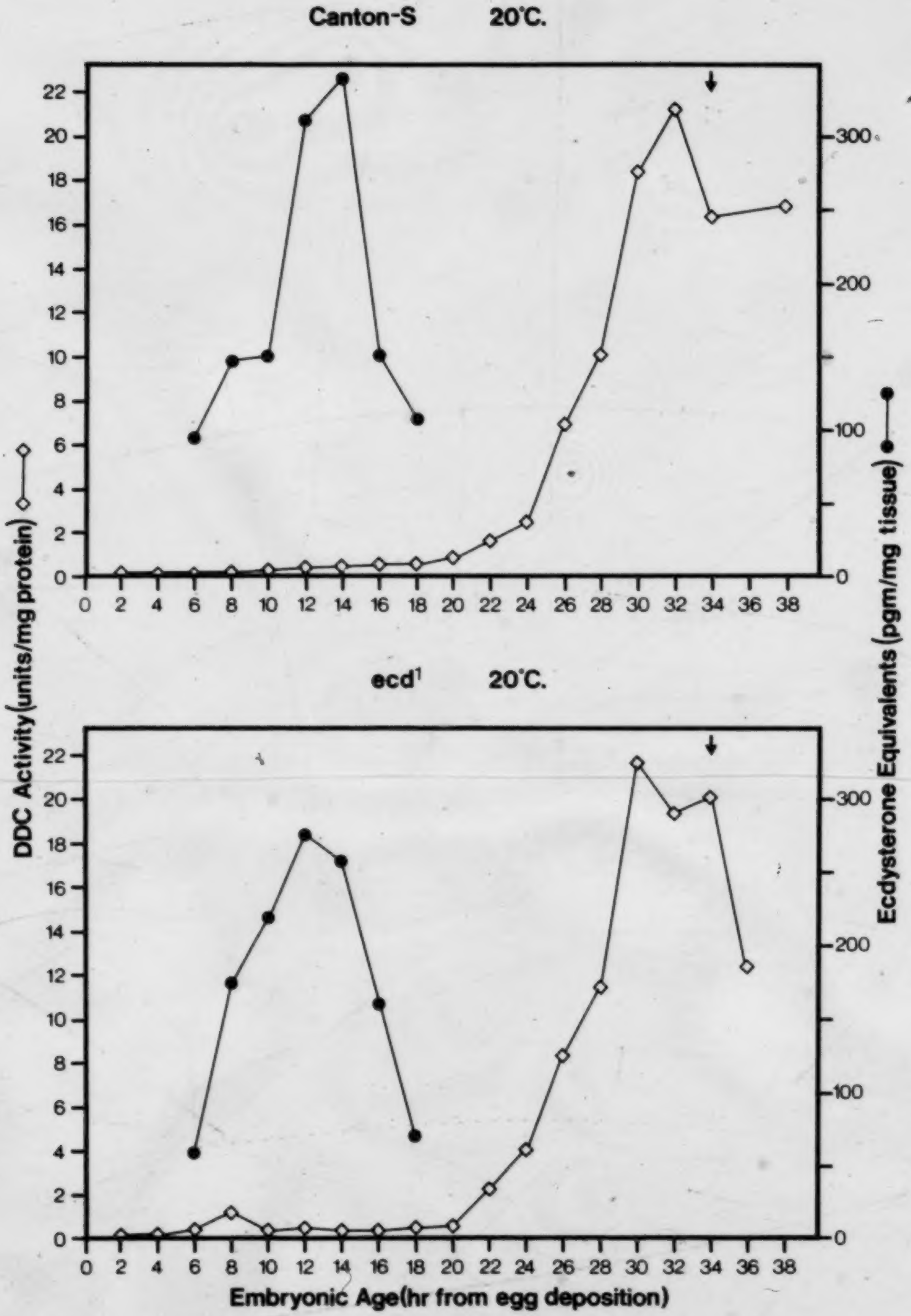
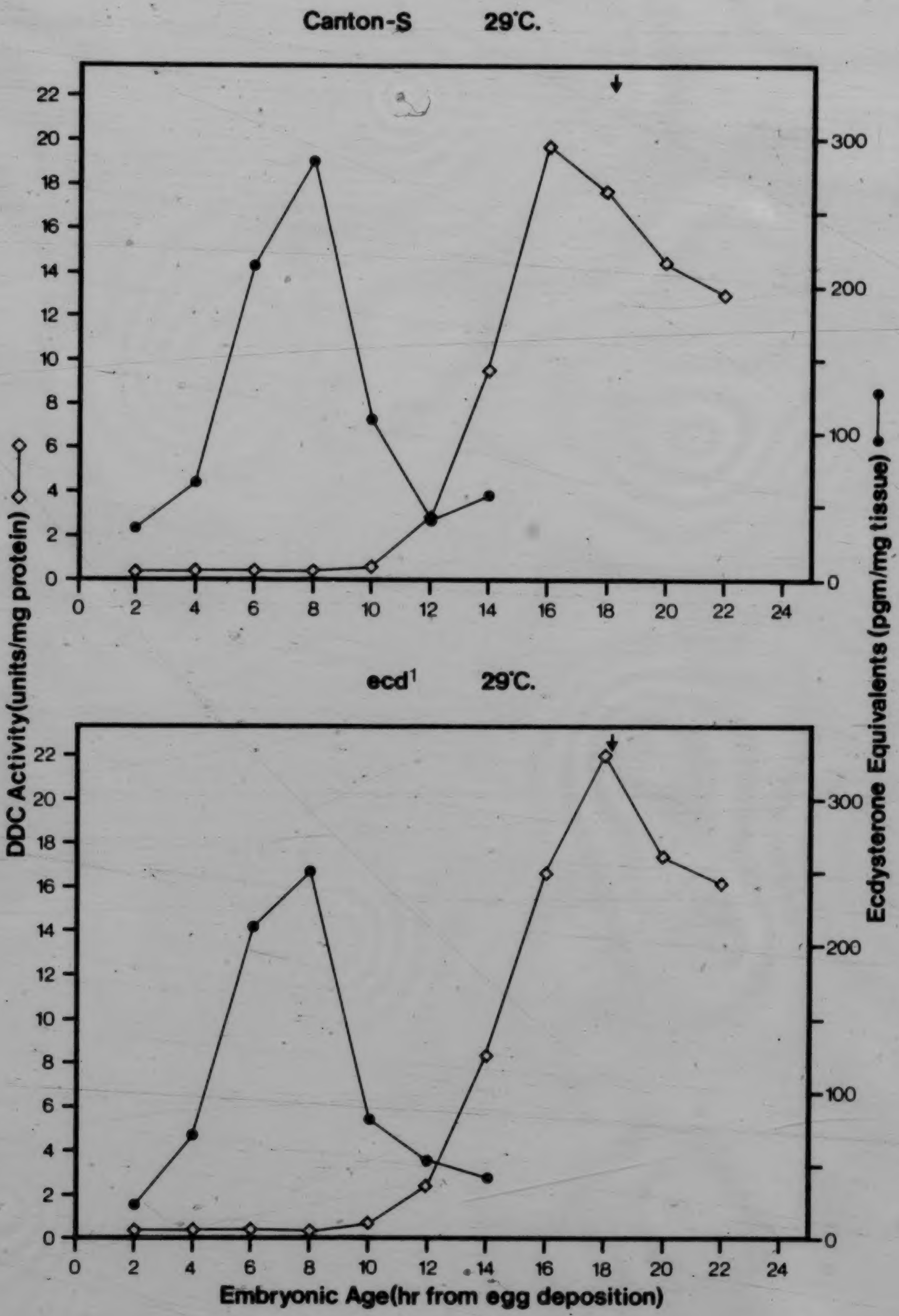

Canton-S 20°C.
ecd1 20°C.
DDC Activity (units/mg protein) ◇—◇
Ecdysterone Equivalents (pgm/mg tissue) ●—●
0
2
4
6
8
10
12
14
16
18
20
22
100
200
300
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38
Embryonic Age (hr from egg deposition)

Figure 4: *Canton-S* and *l(3)ecdysoneless-1*^{ts} DDC activity and ecdysteroid titer measurements during embryogenesis at 29°C.

Embryos from both *Canton-S* and *l(3)ecdysoneless-1*^{ts} strains were collected at 20°C then allowed to develop at 29°C. DDC activity and ecdysteroid titer measurements were again done as in Figure 2. The hatching time of the first larval instar is indicated by the downward arrow.


Canton-S 29°C.
ecd¹ 29°C.
DDC Activity(units/mg protein) ◇—◇
Ecdysterone Equivalents (pgm/mg tissue) ●—●
Embryonic Age(hr from egg deposition)
0
2
4
6
8
10
12
14
16
18
20
22
24
100
200
300

investigated. It was hoped that this mutant could be used as a tool in analysing the regulation of DDC gene expression at this stage.

Figure 3 shows the embryonic ecdysteroid titers and DDC activity measurements of the *Canton-S* wild-type strain and the *l(3)ecdysoneless-1*'' strain maintained at 20°C. This demonstrates that at the permissive temperature the two strains are indistinguishable.

Any effects that *ecd*' might exhibit during embryogenesis at the restrictive temperature were investigated using embryos collected at 20°C for 2 hr, then incubated at 29°C for the desired period. This method of collection was necessary because *l(3)ecdysoneless-1*'' females are sterile when maintained at 29°C (Garen *et al*, 1977). Figure 4 demonstrates that even at the restrictive temperature both strains are similar. From this analysis it seems that the *l(3)ecdysoneless-1*'' mutation has no apparent effect on either the embryonic ecdysteroid accumulation, or the onset of DDC activity. For this reason, all further analyses were performed with the wild-type strain *Canton-S*.

IDENTIFICATION AND QUANTIFICATION OF EMBRYONIC ECDYSTEROIDS

It seemed possible that the different temporal relationship between the ecdysteroids and the onset of DDC activity during embryogenesis could reflect an altered interaction between them. This could be brought about if the embryonic ecdysteroids detected by RIA differed from those found during pupariation. Numerous ecdysteroid types have been described in many different systems (Goodwin *et al*, 1978). Thus the embryonic ecdysteroid peak was analysed to determine in what amounts the puparial ecdysteroids, ecdysone and 20-OH ecdysone, were present.

All TLC and HPLC analysis was again done by Dr. J. D. O'Connor from U.C.L.A.. The ecdysteroids found in 10-12 hr (25°C) *Canton-S* embryos are predominately ecdysone and 20-OH ecdysone. Figure 5a demonstrates the RIA activity of the TLC zones from the chromatographed sample. The ecdysteroids were quantified by elution from TLC zones followed by high performance liquid chromatography (Figure 5b,c). The relative amounts of 20-OH ecdysone and ecdysone were 1.86:1 respectively. In Figure 5a we also see a substantial amount of RIA positive material migrating to the 10cm zone in the chromatograph. Compounds with similar Rf's have been seen by Lagueux *et al* (1979) in *Locusta* embryos and were identified as deoxyecdysone and dehyroxyecdysone. Also in Figure 5a, no RIA positive material is visualized at the origin of the TLC plate. In *Locusta* compounds with similar migration rates have been shown to be polar

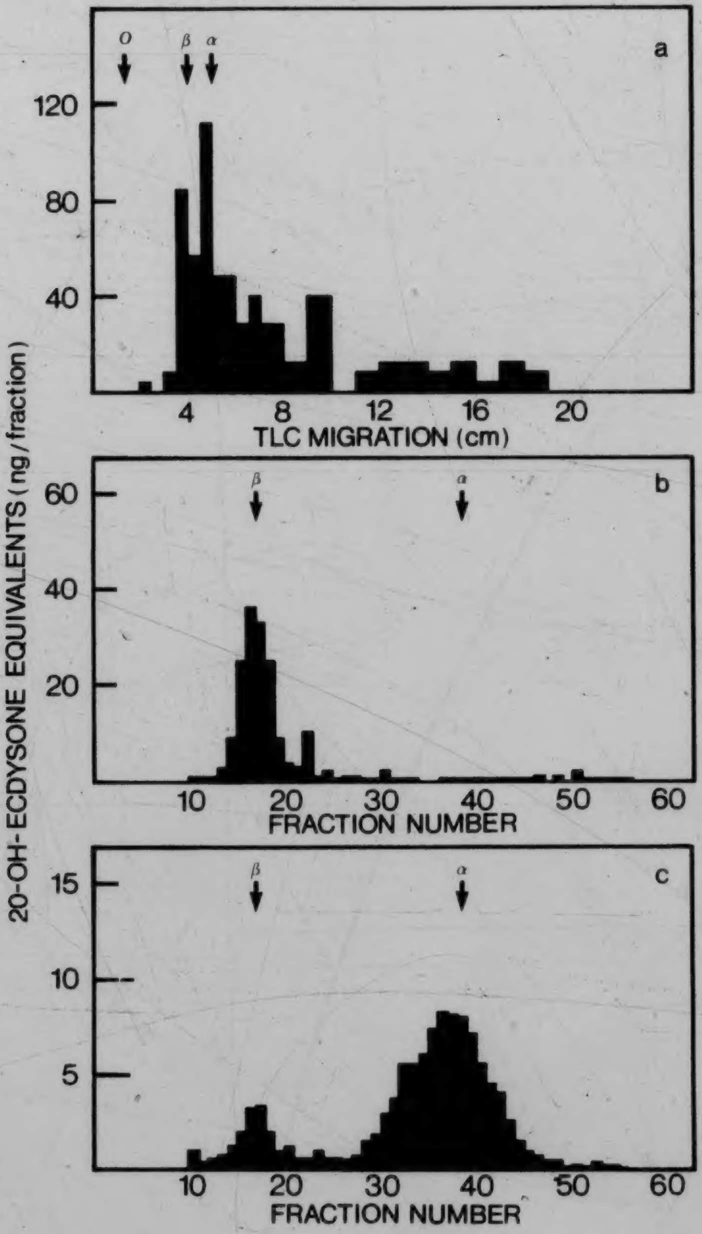
Figure 5: Identification and quantification of the major embryonic ecdysteroids.

All ecdysteroid analysis was kindly done by Dr. J.D. O'Connor (U.C.L.A.). A three gram sample of 10-12 hr (25°C) *Canton-S* embryos was extracted for ecdysteroids as described in the Materials and Methods.

A) TLC separation of RIA active material in 10-12 hr embryo sample.

B)HPLC separation of the TLC material comigrating with an authentic 20-OH ecdysone standard (TLC zone = 3.5-4.5 cm (a)).

C)HPLC separation of the TLC material comigrating with an authentic ecdysone standard (TLC zone = 4.5-6.0 cm (a)). Arrows designate the TLC or HPLC chromatographic behaviour of the 20-OH ecdysone (β) and ecdysone (α), while "O" marks the origin of the TLC chromatograph.


20-OH-ECDYSONE EQUIVALENTS (ng/fraction)
a
o
β
α
120
80
40
4
8
12
16
20
TLC MIGRATION (cm)
b
β
α
60
40
20
10
20
30
40
50
60
FRACTION NUMBER
c
β
α
15
10
5
10
20
30
40
50
60
FRACTION NUMBER

conjugates of 20-OH ecdysone (Lagueux *et al*, 1979). Their absence is considered to be genuine as the sera used in this analysis has been shown to identify such compounds in *Locusta* (B. Sage personal communication). Thus, the major embryonic ecdysteroids were found to be identical to those present during pupariation; therefore the investigation continued in an attempt to determine the cause of the temporal lag between the embryogenesis hormone peak and the expression of the DDC gene.

IDENTITY OF EMBRYONIC DDC

The identity of embryonic DDC was tested to determine if it was produced from the same structural gene responsible for the puparial enzyme. The DDC from embryos, white prepupae, and newly eclosed adults was tested using immunological and genetic methods for homology.

Immunological Analysis

Clark *et al* (1978) first purified the third instar larval DDC protein and produced monospecific anti-DDC antibody. This allowed me to test the specificity of the antibody against the DDC activity in an embryonic crude extract. (Table 1). The inactivation of the embryonic enzyme activity suggests similarity to the larval protein. Immunological similarity between the embryonic, the third larval instar, and the adult enzyme was confirmed by immunodiffusion analysis. Ouchterlony plates were developed

Table 1: Embryonic DDC immunoreactivity.

A wandering third instar larval extract and a late embryonic extract were both made at a concentration of 100mg tissue/ml as described in Clark *et al* (1978). Samples were diluted with anti-DDC IgG and incubated for 2.5 hours on ice. The immunecomplexes were pelleted by centrifugation at 12000 xg for 20 min at 4°C. The supernatant was then assayed for DDC activity as described in Clark *et al* (1978).

TABLE 1

EMBRYONIC DDC IMMUNOREACTIVITY

	DDC ACTIVITY (UNITS)	
SAMPLES	**EMBRYOS**	**LARVAE**
Extract DDC Activity	0.670	0.586
2% IgG Diln	0.036	0.020
4% IgG Diln	0.012	0.010

One unit = 1 nanomole L-dopa decarboxylated/hr/mg tissue

Figure 6: Immunodiffusion analysis of embryonic DDC. Double immunodiffusion was performed as described in the Materials and Methods. Embryonic (E), wandering third instar larval (L), and newly eclosed adults (A) postmitochondrial extracts where placed in the outer wells and ^{125}Iodine labelled anti-DDC IgG (2.88μg, 0.223μCi/μg) was placed in the center well. The autoradiogram was exposed for 8 days at -40°C.

using crude extracts and the results are shown in Figure 6. The smooth geometry of the precipitin line indicates good immunological homology between antigens at all three stages (Kabat, 1976).

Genetic Analysis

In order to investigate the genetic similarity between the DDC activities found during embryogenesis, pupariation, and adult eclosion the temperature-sensitive lethal mutation *l(2)Ddc*^{ts2}, isolated and characterized by Wright *et al* (1981), was employed. These authors have shown that this mutation decreases DDC activity at adult eclosion to approximately 2% of the wild-type values. Recently, Wright (personal communication) has shown that the DDC activity in a *ts*^{2} crude extract from young adult tissue incubated at 56°C decays at a much faster rate than the DDC activity in a wild-type preparation. This result was also recently reproduced by R.B. Hogdetts using a recombinant strain produced by the genetic mapping. This is strong evidence that the lesion in the *l(2)Ddc*^{ts2} strain resides in the structural region of the gene.

Table 2 shows a comparison of DDC activities between *Canton-S* and mutant *l(2)Ddc*^{ts2} strains at the three stages. During embryogenesis, as well as at pupariation and adult eclosion, the *ts*^{2} mutation substantially reduces the DDC activity below that found in *Canton-S*. This result taken together with the evidence from the immunological analysis

Table 2: *l(2)Ddc*^{ts2} DDC activity.

Late embryonic, white prepupal, and newly eclosed adults of both *Canton-S* and *l(2)Ddc*^{ts2} strains were assayed for DDC activity basically as described by Clark *et al* (1978). All embryonic extracts were made at a concentration of 100mg/ml while all other extracts were made at a concentration of 50mg/ml. All *l(2)Ddc*^{ts2} DDC assays were performed on 100μl of extract while the *Canton-S* DDC assays were done with 50μl of sample.

TABLE 2

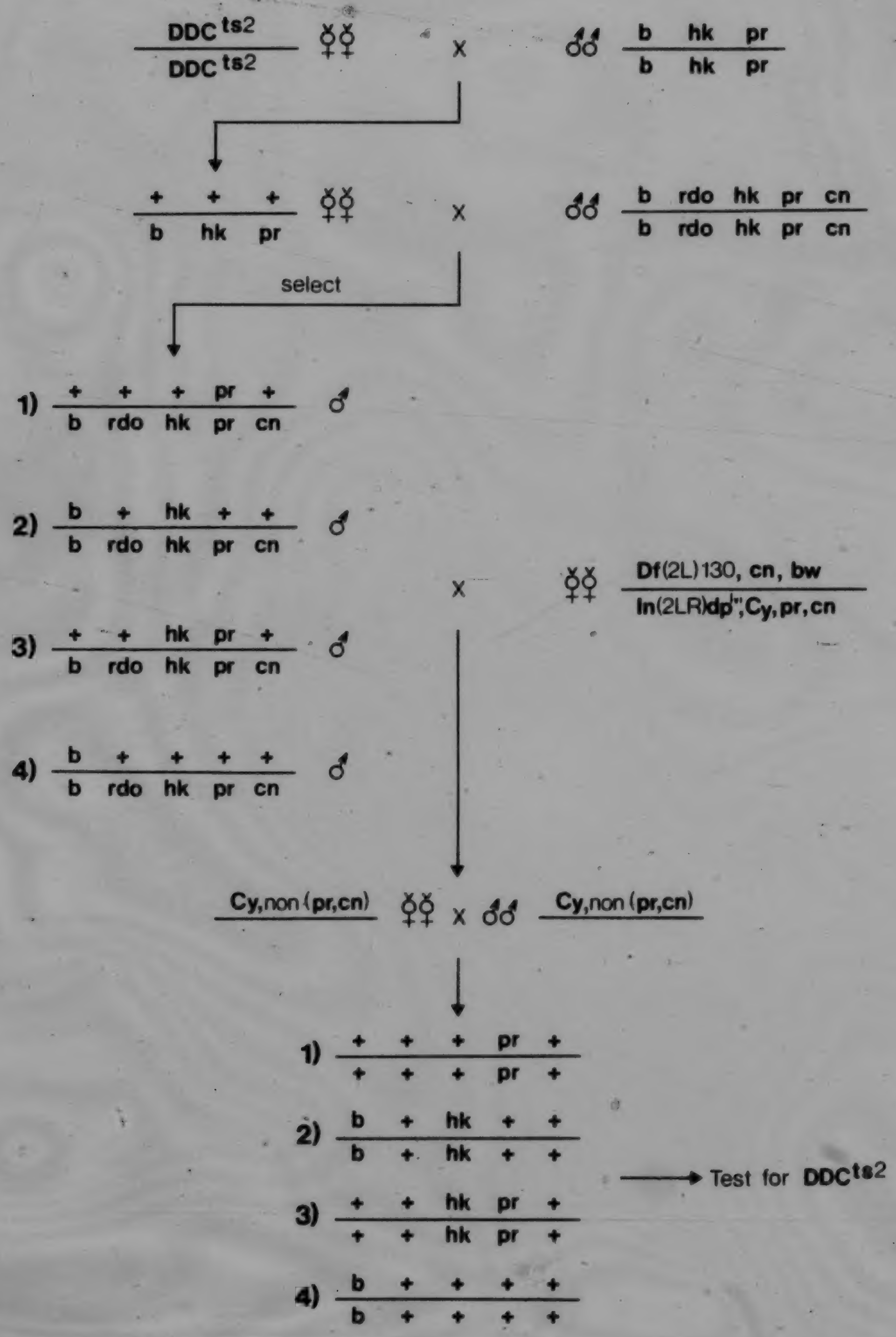
DDC ACTIVITY IN *Canton-S* AND $l(2)Ddc^{ts2}$

	DDC ACTIVITY (UNITS)		
	C-S	ts^2	*ratio*
EMBRYOS	0.572	0.016	0.027
WHITE PREPUPAE	3.048	0.056	0.018
NEWLY ECLOSED ADULTS	3.536	0.080	0.022

One unit = 1 nanomole L-dopa decarboxylated/hr/mg tissue

Figure 7: Genetic mapping scheme used to position *l(2)Ddc*^{ts2}.

This mapping scheme was used to determine if the *l(2)Ddc*^{ts2} mutation mapped to the position of the DDC structural gene locus between the genetic markers *b* and *pr*, (Wright *et al*, 1976). The presence of the *l(2)Ddc*^{ts2} allele in all recombinants tested was determined by its lethal phenotype during pupariation when raised at 29°C. All strains used in these crosses were those resident to R.B. Hodgetts' laboratory.


DDC ts2 / DDC ts2 ☿☿ x ♂♂ b hk pr / b hk pr
+ + + / b hk pr ☿☿ x ♂♂ b rdo hk pr cn / b rdo hk pr cn
select
1) + + + pr + / b rdo hk pr cn ♂
2) b + hk + + / b rdo hk pr cn ♂
3) + + hk pr + / b rdo hk pr cn ♂
4) b + + + + / b rdo hk pr cn ♂
x ☿☿ Df(2L)130, cn, bw / In(2LR)dp^lv,Cy,pr,cn
Cy,non (pr,cn) ☿☿ x ♂♂ Cy,non (pr,cn)
1) + + + pr + / + + + pr +
2) b + hk + + / b + hk + +
3) + + hk pr + / + + hk pr +
4) b + + + + / b + + + +
Test for DDCts2

strongly suggest that the DDC activity at these stages is produced from the same structural gene.

Finally the genetic map position of the *ts*^{2} lesion was determined using recombination analysis. This was done to rule out the possibility that a trans-acting regulatory modifier was causing the decrease in DDC activity in the original mutant strain. Wright *et al* (1976) located the DDC structural gene between the genetic markers *hk* and *pr* on the second chromosome. Figure 7 displays the scheme I used to determine if the *l(2)Ddc*^{ts2} mutation was also located at this position. From these crosses four types of recombinants were recovered and analysed. The recombination events between the genetic markers *b* and *hk* produced many *b++* and *+hkpr* recombinants. Three of each type were analysed for the lethal phenotype produced at 29°C by the *ts*^{2} mutation. All three *b++* recombinants produced this lethal phenotype indicating the presence of the *ts*^{2} mutation. None of the *+hkpr* recombinants proved to harbour the mutation. This suggests that the location of *l(2)Ddc*^{ts2} is close to the marker *hk*. Recombination events between *hk* and *pr* produced ten *++pr* and nine *bhk+* recombinants. Only eight of the *++pr* strains produced the *ts*^{2} lethal phenotype at 29°C. The three tested for DDC activity at the adult stage were identical to the original *ts*^{2} allele. All nine *bhk+* recombinants when tested for the *ts*^{2} lesion did not show the *ts*^{2} lethal phenotype. These results show that the *ts*^{2} mutation is situated between the genetic markers *hk* and *pr*. The relative location of the ts^2 mutation can be calculated from the data using a standard mapping formula: map position= $Z+(a/a+b)x$, where Z is the map position of the marker *hk*, x is the number of map units between *hk* and *pr*, a is the number of recombination events between *hk* and the ts^2 mutation, and b is the number of events between the ts^2 mutation and the marker *pr*. This makes Z= 53.9, x= 0.6, a= 2, and b= 17. The map position of $l(2)Ddc^{ts2}$ is calculated to be 53.96 which falls between 54.1 and 53.295 reported by Wright *et al* (1976, 1981 respectively) for the Ddc structural gene.

Thus, from these analyses it seems that the temporal lag between the embryonic hormone peak and onset of DDC activity is not attributable to the embryonic enzyme being produced by a gene distinct from that responsible for the puparial DDC activity.

DDC GENE EXPRESSION DURING EMBRYOGENESIS

The previous results suggest that, the ecdysteroids, and the DDC gene product present during embryogenesis are identical to their puparial counterparts. Given this, it is possible that the relationship between the ecdysteroid titer peak and the DDC gene during embryogenesis is similar to that at pupariation; however, the interaction of other factors could produce the temporal lag in the appearance of the DDC activity. Therefore, I investigated the temporal relationship between the hormone titer peak and the accumulation of DDC gene transcript during embryogenesis.

This was done by analysing RNA extracted from embryos, collected to represent time points every 2 hr throughout embryogenesis, using Northern gel blotting techniques. A DDC clone, kindly provided by Dr. J. Hirsh (Hirsh and Davidson,1981), was used to identify transcripts from the DDC gene. A restriction map for the DDC gene DNA fragment can be found in Figure 8 (Hirsh and Davidson, 1981; J. Hirsh and R.B.Hodgetts personal communications.) The DDC transcript is positioned above the map as indicated in Scholnick *et al* (1983) and the DNA restriction fragments used as probes in this study are shown below the map.

Hybridization Arrested Translation

Upon receipt of a plasmid containing a 4.8kb *EcoR1* DNA fragment I decided to test it for homology to DDC mRNA from third instar larvae. Sucrose gradient fractionated polyadenylated mRNA isolated from induced *l(3)ecdysoneless-1*^{ts} larvae was used due to the high concentrations of translatable DDC mRNA necessary for the HART assay. Figure 9 displays SDS-PAGE of *in vitro* translation products and immunoprecipitable DDC from the sucrose gradient fractions. Fraction 22 was used to test the purified 4.8kb *EcoR1* restriction fragment shown in Figure 10. Figure 11 shows that 1.5μg of the 4.8kb DNA (slot A4) completely eliminated immunoprecipitable DDC from the *in vitro* translation products. Compared with one microgram of pBR322 DNA (slot A2) 0.3μg of the 4.8kb fragment (slot A3)

Figure 8: Restriction map of the DDC gene.

Restriction site information was obtained from Hirsh and Davidson (1981) and Drs. J. Hirsh, and R.B. Hodgetts (personal communications). The location of the DDC transcript was positioned as reported in Scholnick *et al* (1983). The large solid blocks represent exonic regions and the broken lines represent intron sequences. The DNA probes referred to during the course of this work are indicated by solid lines below the restriction map. The artificial *EcoR1* site is designated '*' and was generated by the original cloning of randomly sheared DNA with *EcoR1* linkers (Hirsh and Davidson, 1982).

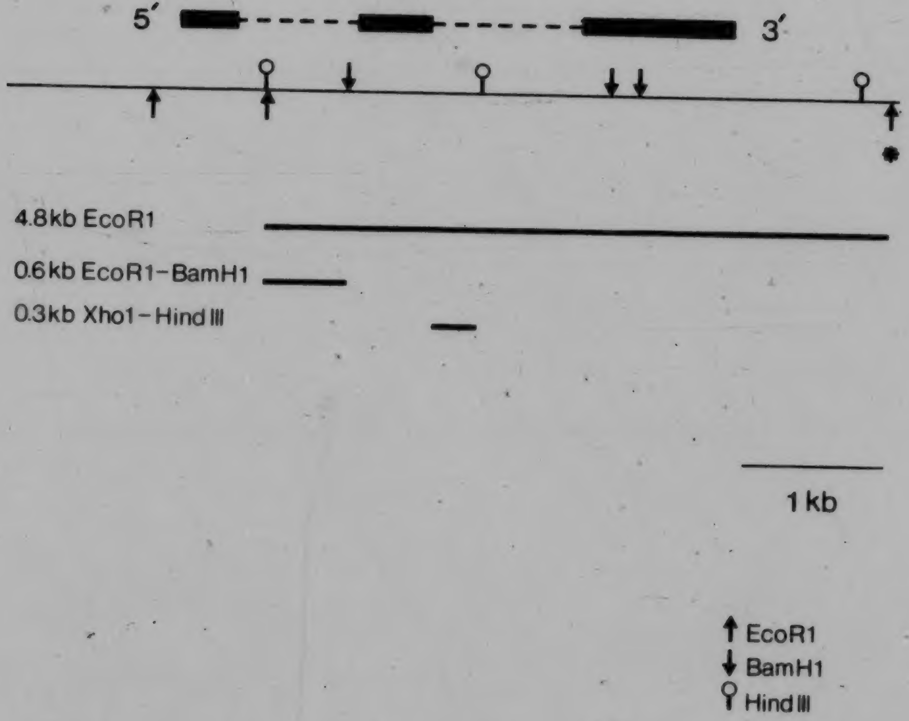

5′
3′
4.8 kb EcoR1
0.6 kb EcoR1–BamH1
0.3 kb Xho1–Hind III
1 kb
EcoR1
BamH1
Hind III

Figure 9: Size fractionation of larval poly(A) RNA. Three hundred micrograms of poly(A) mRNA isolated from *l(3)ecdysoneless-1*^{ts} larvae induced with 20-OH ecdysone as described by Kraminsky *et al* (1980) was fractionated using sucrose gradient centrifugation as described in the Materials and Methods. The recovered RNA from each fraction was translated *in vitro* in a volume of 50μl. The total translation products were analysed on a SDS 10% acrylamide gel loading 1.8 x 10^5 cpm/slot, (Total). Each fraction was immunoprecipitated from 7.2 x 10^6 cpm of ^{35}S-methionine incorporation and also gel analysed as done with the total products. The immunoprecipitations were carried out with anti-DDC IgG. Each gel was fixed in 10% acetic acid and prepared for fluorography as described in the Materials and Methods. Each fluorogram was exposed for 1 day at -40°C. The sucrose gradient fraction numbers are indicated below each slot and the molecular weight indicated is that of the major mRNA dependent lysate translation product.

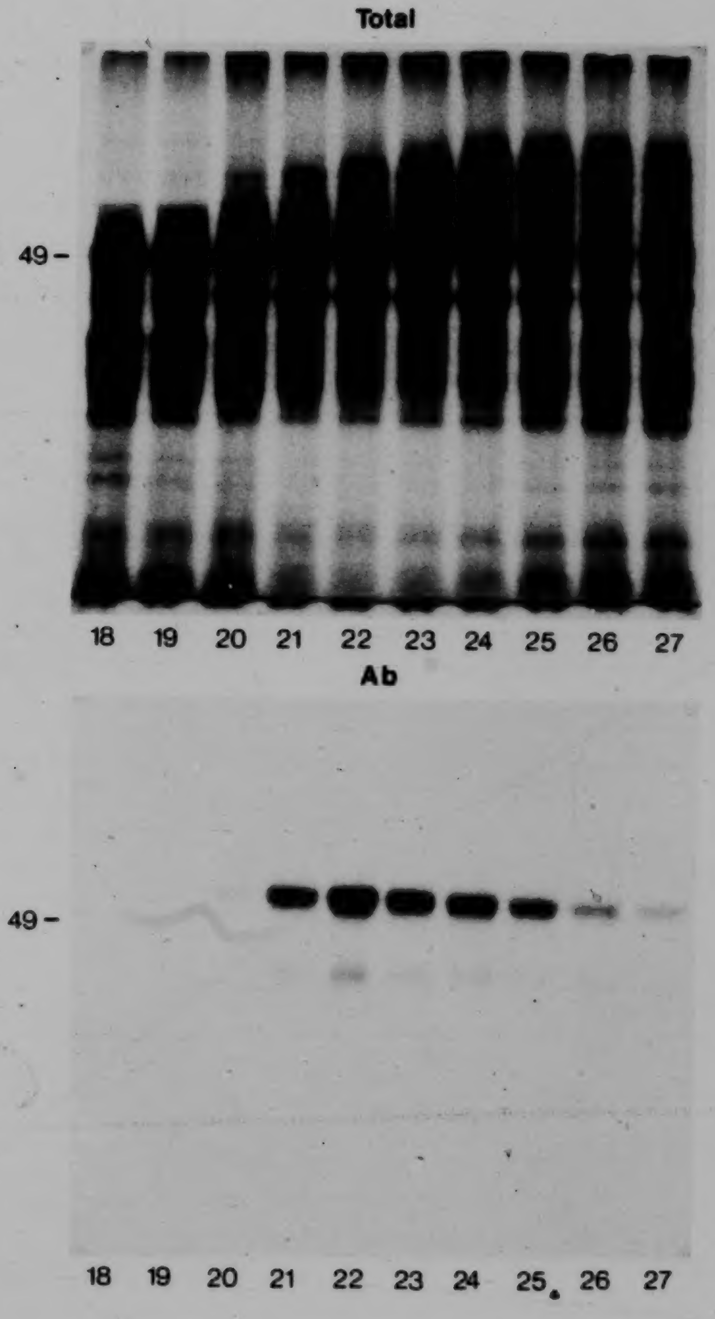

Total
49 -
18 19 20 21 22 23 24 25 26 27
Ab
49 -
18 19 20 21 22 23 24 25 26 27

Figure 10: DNA restriction fragment purification.

The DNA fragments used as probes in northern blot analyses were purified by electroelution or by phenol extraction as described in the Materials and Methods.

Figure 10A represents a 0.8%(w/v) agarose gel loaded with plasmid (pACYC184), containing the 4.8kb *EcoR1* DDC cloned fragment, digested with *EcoR1* (slot 1), and 2.5µg (slot 2) and 1.25µg (slot 3) of 4.8kb *EcoR1* DDC DNA fragment purified by electroelution.

Figure 10B represents a 5% acrylamide TAE gel containing the 0.6kb *EcoR1-BamH1* DDC fragment bearing plasmid (pBR322) digested with *EcoR1* and *BamH1* (slot 1), and 0.5µg of 0.6kb *EcoR1-BamH1* DDC DNA fragment (slot 2) purified by phenol extraction of low gelling temperature agarose. In both A and B the gel slot origin is designated, ori. For a restriction map of the DDC gene see Figure 8.

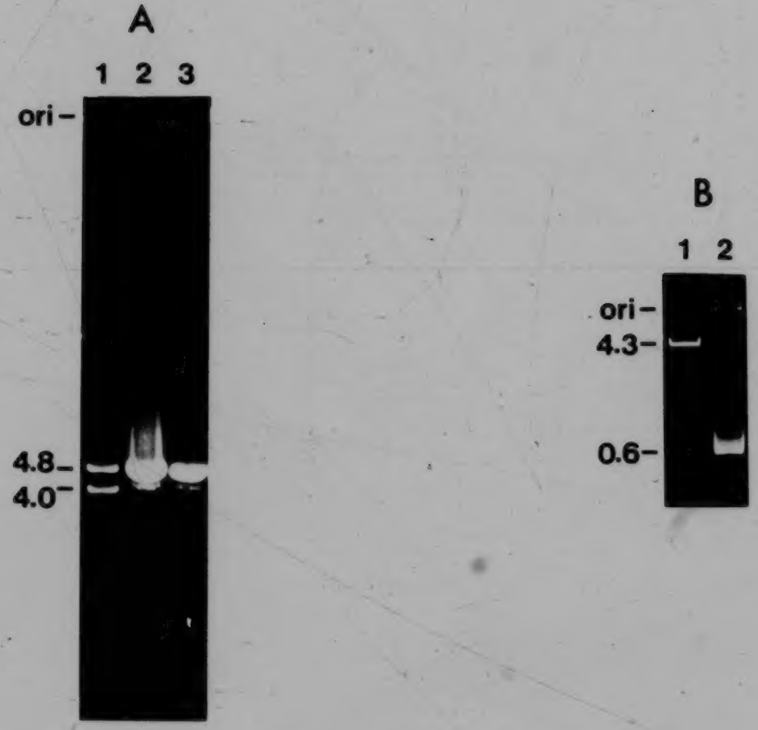
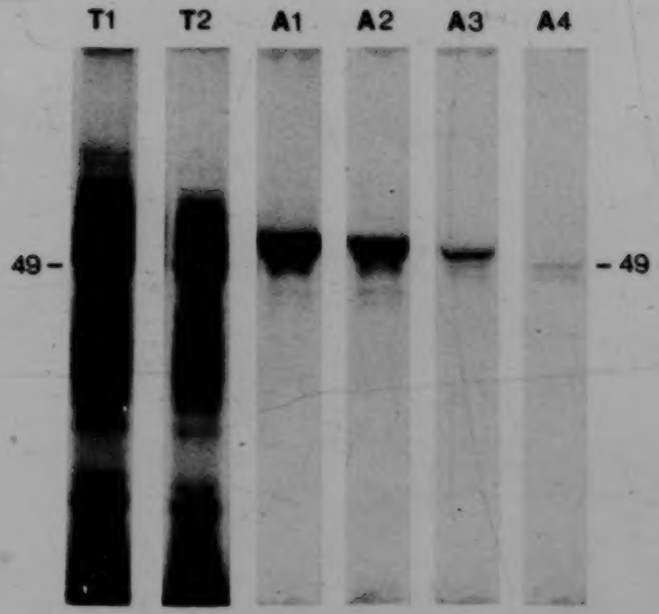

A
1 2 3
ori
4.8
4.0
B
1 2
ori
4.3
0.6

Figure 11: Hybridization arrested translation.

The sequence homology of the 4.8kb *EcoR1* DNA fragment to DDC mRNA was tested. Four micrograms of RNA from fraction 22 (Figure 10) was hybridized to denatured 4.8kb *EcoR1* fragment DNA and pBR322 plasmid DNA. The mixture was then *in vitro* translated and the products subjected to immunoprecipitation. These were then analysed on a SDS 10% polyacrylamide gel and fluorographed. The total products from the unhybridized RNA sample (T1) and the RNA sample after hybridization to 1.5µg of the 4.8kb *EcoR1* fragment DNA (T2) are shown along with the immunoprecipitates from the untreated RNA sample (A1), the RNA sample treated with 1.0µg of pBR322 plasmid DNA (A2), the RNA sample treated with 0.5µg of 4.8kb *EcoR1* fragment DNA (A3), and the RNA sample treated with 1.5µg of the 4.8kb *EcoR1* fragment DNA (A4). The molecular weight indicated is that of the major mRNA dependent lysate translation product. The untreated RNA immunoprecipitate was derived from 3.6×10^6 cpm ^{35}S-methionine incorporation while all others where produced from approximately 1.2×10^6 cpm ^{35}S-methionine incorporation.

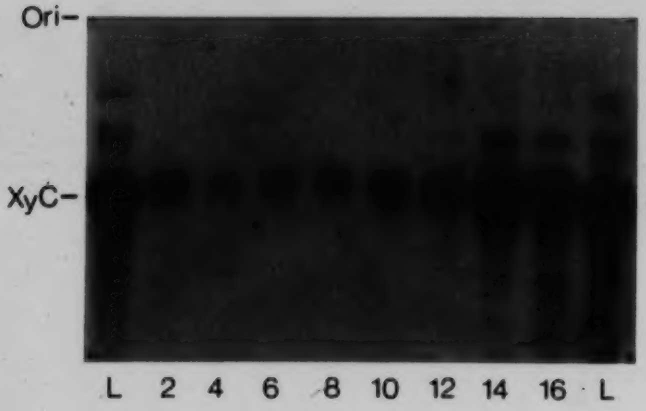

T1
T2
A1
A2
A3
A4
49 -
- 49

also noticeably reduced the amount of DDC immunoprecipitate. The reduced amount of immunoprecipitate in the pBR322 sample compared to the control sample (slot A1) is due to the reduction of mRNA translatability after mRNA samples are incubated at hybridization temperatures. Thus, these findings confirm the results of Hirsh and Davidson (1981) proving this 4.8kb *EcoR1* DNA fragment's utility as a probe in Northern gel blotting analyses.

Northern Blot Analysis

Canton-S embryo samples were collected to represent time points every two hours throughout embryogenesis at 29°C. This temperature was used to reduce the number of samples which had to be analysed. The polyadenylated mRNA was isolated from each sample by phenol extraction and oligo-dT chromatography. This mRNA was electrophoresed using a horizontal submarine gel apparatus in a denaturing formaldehyde agarose gel and blotted to nitrocellulose paper which was then hybridized to nick translated 4.8kb *EcoR1* DNA. Figure 12 displays a resultant autoradiogram. From this analysis we see that transcripts with homology to the 4.8kb *EcoR1* DDC probe occur in both early and late embryonic RNA samples. In the 2 hr sample a transcript, slightly larger than the major response in the larval samples, is visualized. By 4 hr of development this transcript has disappeared and no major accumulation of DDC transcript occurs until 14-16 hr. Thus, from these results it seems

Figure 12: DDC transcript accumulation during embryogenesis. Two micrograms of poly(A) RNA extracted from *Canton-S* embryo samples representing time points throughout embryogenesis at 29°C were electrophoresed in a submarine 1.5%(w/v) agarose northern gel, blotted to nitrocellulose, and probed with 4.8kb *EcoR1* DDC DNA fragment as described in the Materials and Methods. The late third instar larval samples are designated, L, and the mean developmental time of each embryo RNA sample is indicated. The gel origin is designated, ori, and the position of the xylene cyanol dye front, XyC. In this gel system the xylene cyanol dye front migrates with 2 kb transcripts. The autoradiogram was exposed for 2 days at -70°C.


Ori–
XyC–
L
2
4
6
8
10
12
14
16
L

that the embryonic hormone peak, occuring at 8 hr does not immediately stimulate the production of transcripts from the DDC gene. From this it seems clear that the temporal relationship between the embryonic hormone peak and DDC expression is similar to the late puffing scenario mentioned earlier.

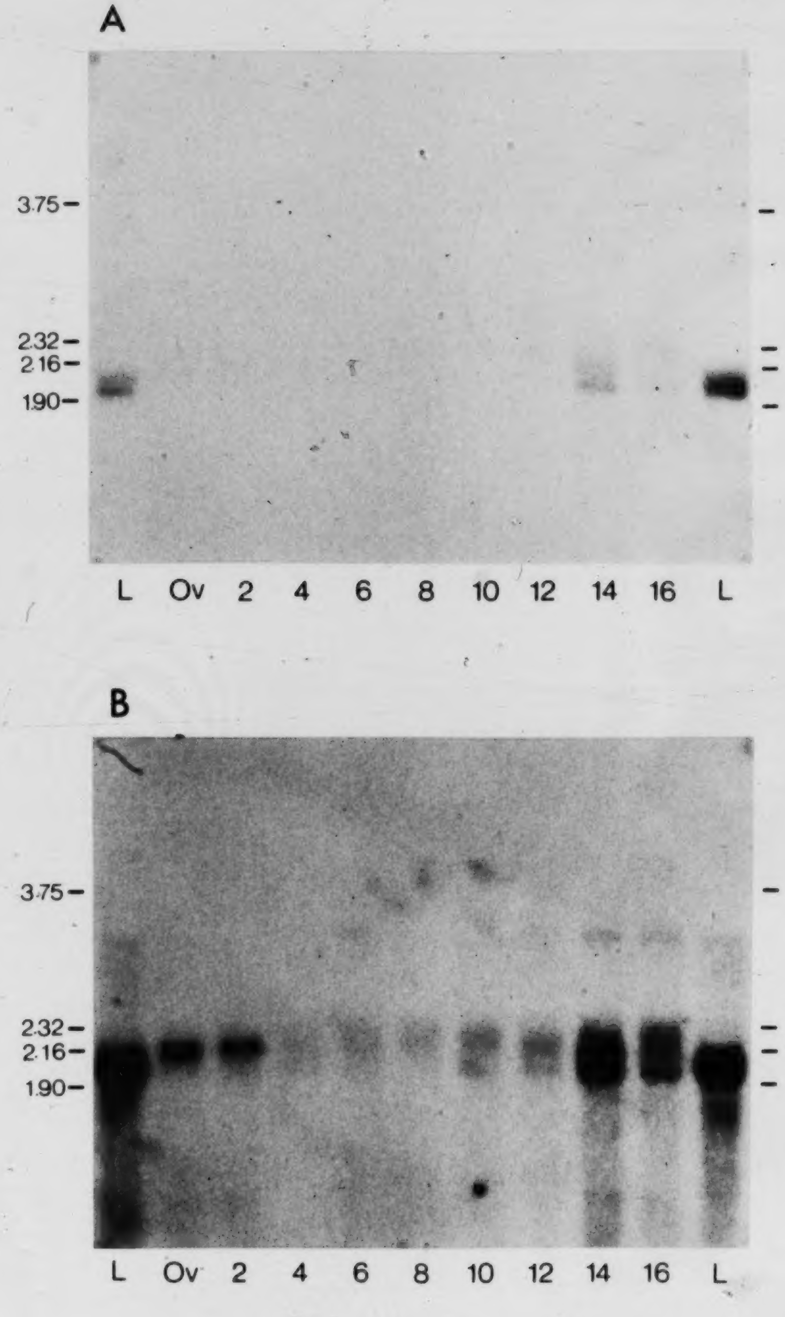
Also noticeable from this Northern gel blot is that the number and the size of the DDC transcripts in the embryonic and larval RNA samples vary. The shape of the late embryonic transcript bands suggests that perhaps several bands were contained within them. For this reason, these RNA samples were analysed using a high resolution Northern gel system. The presence of the DDC transcript in the 2 hr RNA sample suggested that it may be maternally contributed. Therefore a RNA sample extracted from ovaries taken from virgin females was included in this analysis. Figure 13 displays autoradiograms produced using the H:V gel system described in the Materials and Methods. This blot more dramatically shows the independence in the accumulation of DDC transcript during late embryogenesis from the embryonic ecdysteroid peak. We also see that the 2 hr DDC transcript is present in the ovary RNA sample (Figure 13B, slot Ov) suggesting that this early transcript is of maternal origin. This gel blot also shows that the major DDC transcript band seen in both the 12-16 hr and the larval RNA samples is composed of multiple transcript species. The shortest exposure (Figure 13A) clearly demonstrates that in both the larval and the

Figure 13: High resolution northern gel analysis of embryonic DDC transcripts.

Two micrograms of each embryonic RNA sample (Figure 12) was electrophoresed in a (H:V) 1.35% agarose northern gel as described in the Materials and Methods. The samples were electrophoresed until the xylene cyanol dye front had migrated about 5 cm, the gel blotted and then probed with the 4.8kb *EcoR1* DDC DNA fragment as described in the Materials and Methods. The late larval instar RNA samples are designated, L , the ovary RNA sample, Ov, and the mean developmental time each embryonic RNA sample is indicated. The sizes indicated correspond to the migration yeast and *Drosophila* ribosomal RNA samples (see Figure 19).

A) 3 day exposure at -70°C.

B) 8 day exposure at -70°C.


A
3.75
2.32
2.16
1.90
L
Ov
2
4
6
8
10
12
14
16
L
B
3.75
2.32
2.16
1.90
L
Ov
2
4
6
8
10
12
14
16
L

14 hr samples the major band is resolved into a doublet. Figure 13B reveals that in the 16 hr sample another wide band is observed that is larger than the 14hr doublet.

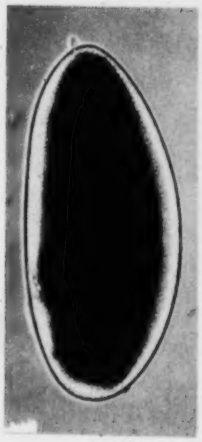
To corroborate these results I decided to extract RNA from another set of embryo samples. During the collection more attention was given to the synchrony of each embryo sample to reduce the number of withheld eggs. Immediately following the 2 hr collection period a small representative sample was dechorionated and examined under low power using a Zeiss microscope equipped with phase-contrast optics. If the preblastoderm embryos represented less than 85% of the sample it was not used for RNA extractions. Typically samples contained greater than 95% of the embryos between the preblastoderm and early gastrula stages. Figure 14 depicts the developmental landmarks during embryonic stages at 29°C. The polyadenylated mRNA was purified from these embryo samples as described except SDS was added to a final concentration of 1%(w/v) before phenol extraction. Thus the mRNA from these samples should represent both nuclear and cytoplasmic species.

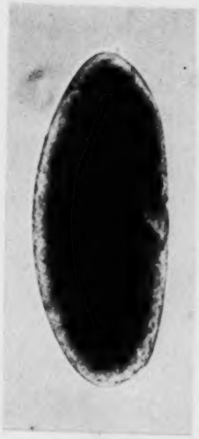
For greater resolution two micrograms of each poly(A) mRNA sample was electrophoresed, using the H:V gel system, until the xylene cyanol dye had migrated approximately 15cm. Figure 15A, B, C show increasing exposures from a northern gel blot using this high resolution gel system. In this northern analysis various sized transcripts are evident in samples containing significant amounts of DDC transcripts.

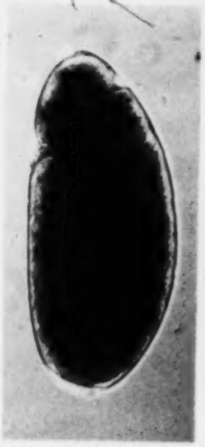
Figure 14: Survey of embryo samples developing at 29°C. The samples collected for the second embryonic RNA extractions were examined in a light microscope to correlate the developmental times at 29°C with stages of embryogenesis. Samples were timed from the midpoint of their 2 hr collection period. All embryos were dechorionated as described in the Materials and Methods then placed on a glass slide in a drop of water under a coverslide. The pictures were taken using a Zeiss binocular microscope equipped with phase contrast optics and a Polaroid camera attachment using type 665 Polaroid film.

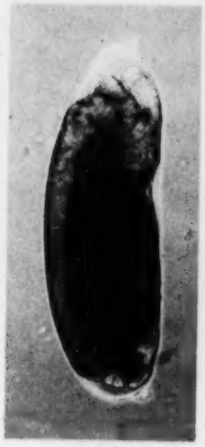
Note added in proof: The 8 hr and 10 hr photographs represent 10 hr and 8 hr embryos respectively.

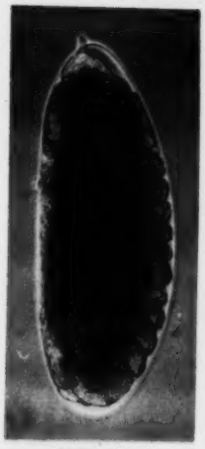
1 hr

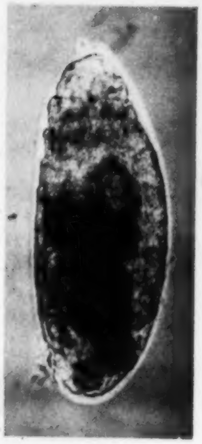

2 hr

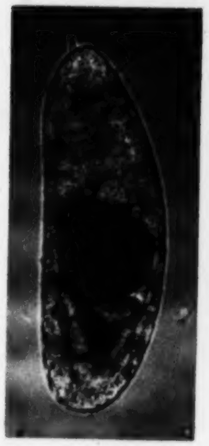

4 hr


6 hr


8 hr


10 hr


12 hr


14 hr

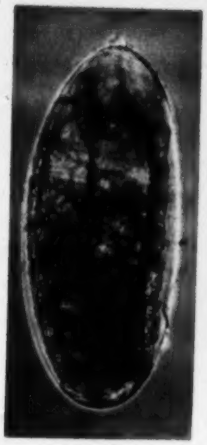

16 hr

Figure 15: High resolution northern gel analysis of embryonic DDC transcripts.

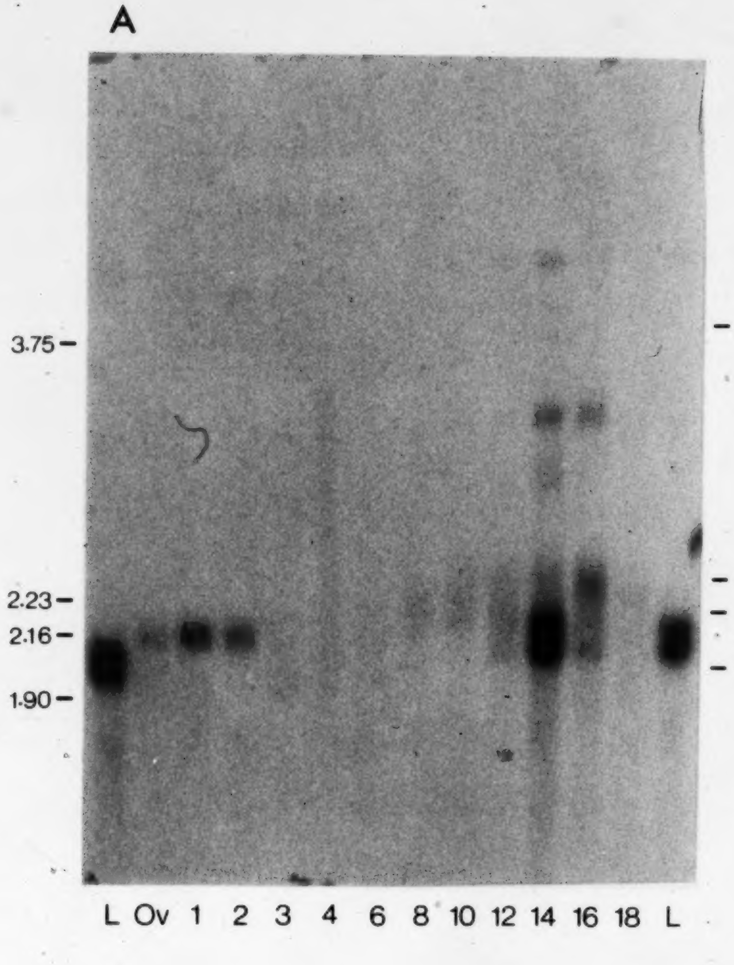
The second set of embryonic samples was prepared to yield total cellular (nuclear and cytoplasmic) poly(A) RNA. Two micrograms of each sample were electrophoresed in a H:V 1.35% agarose northern gel until the xylene cyanol dye front had migrated about 15 cm. The gel was blotted and probed with the 4.8kb *EcoR1* DNA fragment as described in the Materials and Methods. The late third instar RNA samples are designated, L, the ovary RNA, Ov, and the average developmental age of the embryonic RNA samples is indicated. The 18 hr slot represents a newly hatched first instar larval RNA sample.

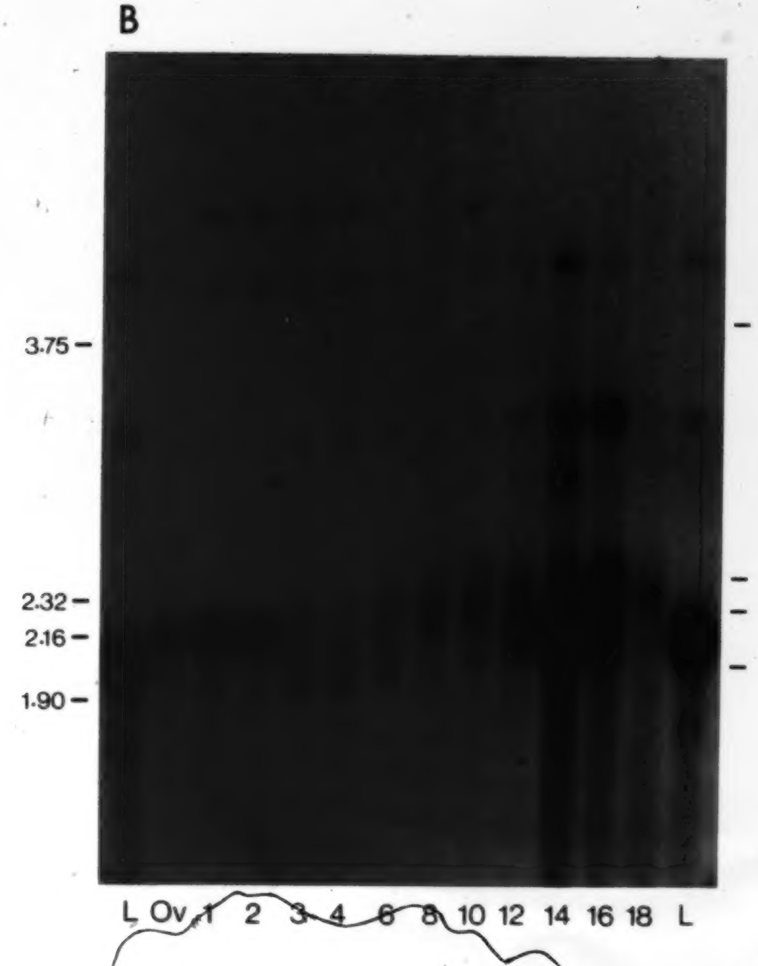
A) represents a 9 hour exposure.

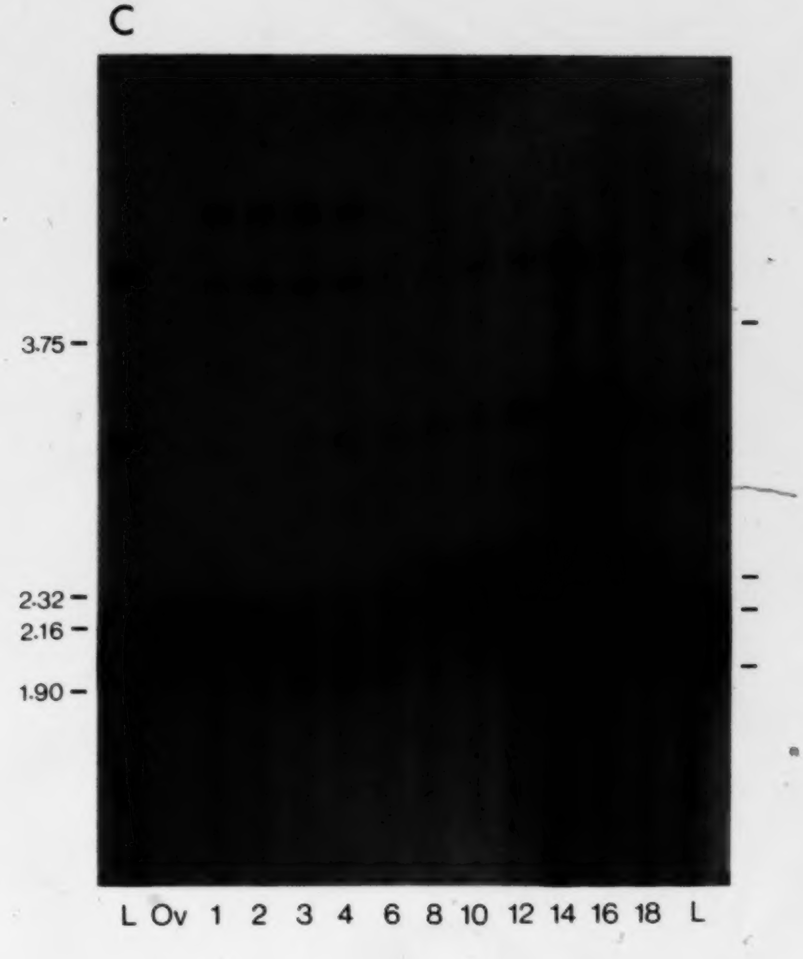
B) represents a 1 day exposure.

C) represents a 2 day exposure.

All exposures were carried out at -70°C.


A
3.75
2.23
2.16
1.90
L
Ov
1
2
3
4
6
8
10
12
14
16
18
L


B
3.75
2.32
2.16
1.90
L
Ov
1
2
3
4
6
8
10
12
14
16
18
L


C
3.75
2.32
2.16
1.90
L
Ov
1
2
3
4
6
8
10
12
14
16
18
L

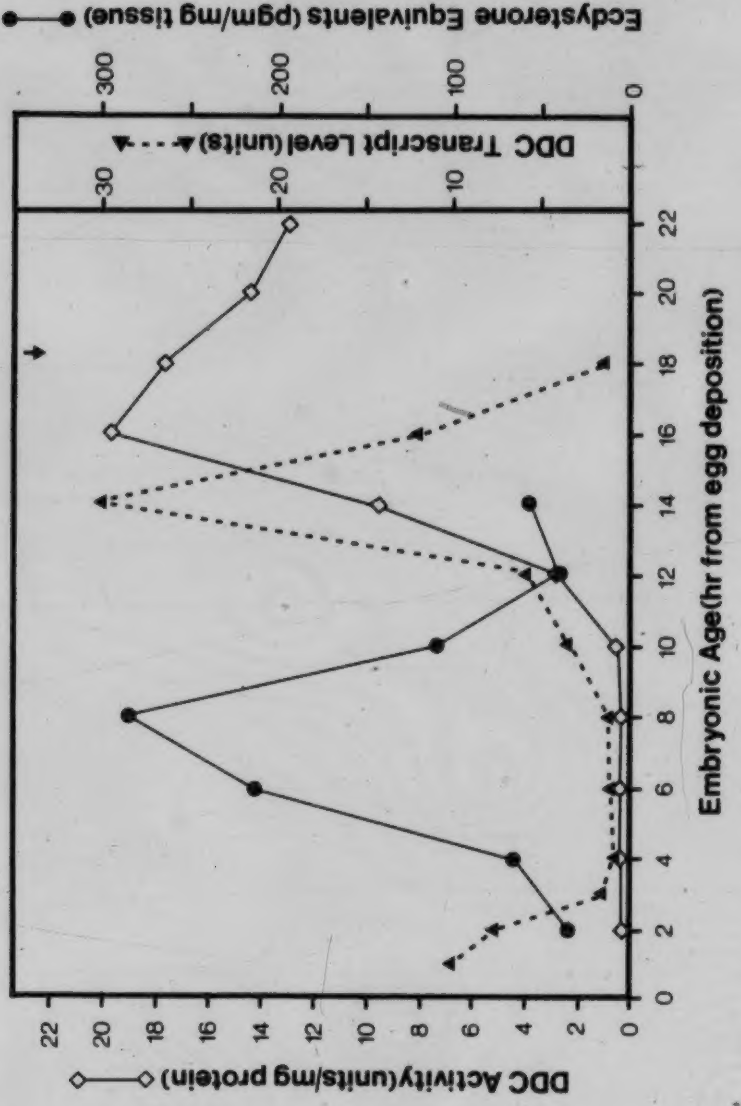
To simplify matters, I would like to focus on the presence of the transcripts about 2kb in size which I believe to represent the fully mature translatable mRNA molecules. In analyses to follow, the relationship between each transcript size with respect to its place in the maturation scheme from primary transcript to translatable mRNA, is addressed. For now, suffice it to say that the transcript's larger than 2.2kb in length represent transcripts not as yet completely processed.

As seen in the previous set of RNA samples there are two periods during embryogenesis where a major accumulation of DDC transcripts occurs (Figure 15A,B). The ovary RNA sample as well as the 1 and 2 hr embryo RNA samples again show a major accumulation of DDC transcript (Figure 15A,B). This corroborates the earlier results adding more evidence to the suggestion that these preblastoderm DDC transcripts are contributed to the developing oocyte by a maternal source. However by 3 hr of development these transcripts have disappeared and little accumulation of DDC transcripts occur until 12-16 hr. This again correlates well to the time DDC activity increases during late embryogenesis.

Figure 16 displays a graph of the ecdysteroid titer and the DDC activity found during embryogenesis at 29°C on which the relative amounts of the 2kb DDC transcripts, estimated by optical density scanning of each autoradiogram gel slot, is also plotted. This clearly demonstrates that DDC transcript accumulation does not occur immediately upon the

Figure 16: The relationship between DDC transcript accumulation and the embryonic ecdysteroid and DDC activity peaks.

The DDC transcripts in the 2.0kb size range visualized in Figure 15 were quantified from an autoradiogram by scanning each gel slot using a Perkin-Elmer spectrophotometer equipped with a gel scanner. The peaks representing the transcripts from 2.0kb to 2.3kb were cut from the gel tracing and weighed. The relative amounts of transcript from each RNA sample were then plotted onto the graph of *Canton-S* embryonic ecdysteroid titer and DDC activity from Figure 4. The hatching time of the first larval instar is indicated by the downward arrow in the upper right corner.


Ecdysterone Equivalents (pgm/mg tissue)
300
200
100
0
DDC Transcript Level (units)
30
20
10
DDC Activity(units/mg protein)
22
20
18
16
14
12
10
8
6
4
2
0
Embryonic Age(hr from egg deposition)

rise in embryonic ecdysteroid titer. In fact, no accumulation of DDC occurs until just prior to the onset embryonic DDC activity. Interestingly, Figure 16 demonstrates that the DDC transcript accumulation begins about 2 hr before the onset of DDC activity and also peaks 2 hr prior to the peak in DDC activity. Also the amount of DDC transcript falls off rapidly, after peaking at 14 hr, to baseline levels at 18 hr suggesting that the half-life of the DDC transcripts made during late embryogenesis is relatively short.

The northern analyses of both sets of embryonic RNA samples have shown that the relationship between the hormone titer peak and the onset of DDC gene expression differs from that seen during pupariation. The temporal lag in the production of DDC gene transcripts after the ecdysteroid peak during embryogenesis suggests that the interaction between the hormone and the DDC gene is similar to the late puffing response to hormone stimulation.

DDC TRANSCRIPT CHARACTERIZATION

In the final part of this thesis I will now focus on analysing the numerous DDC transcripts seen throughout embryogenesis. The major DDC transcript band, previously demonstrated in late embryonic and larval RNA samples (Figure 12) again appears as two distinct transcripts of similar size (Figure 15A). Another transcript species, of slightly larger size, is also visualized in the 12-16 hr RNA

Figure 17: Efficient hybridization to a 14 hr RNA gel blot. The 14 hour poly(A) RNA sample (Figure 15) was electrophoresed in a H:V 1.5% agarose northern gel, blotted, and probed with the 4.8kb *EcoR1* DDC DNA fragment probe as described in the Materials and Methods. The autoradiogram was exposed for 36 hours. Three additional bands between the two largest transcripts are indicated by the i. The size indicated is the position of the 2.0kb DDC transcript (see Table 3).

i
i
i
2.0
14 14

samples (Figure 15A,B). Also in these late embryonic RNA samples, three larger transcripts are noticeable. In the early embryonic RNA samples the major transcript is also accompanied by three larger transcripts, the largest being unique to this stage of embryogenesis (Figure 15C). In Figure 17, which represents another gel blot using the 14 hr RNA sample, three additional diffuse transcript bands of less intensity are visible between the largest transcripts. These bands were evident only in gel blots where the specific activity of the DNA probe was in excess of $4x10^8$cpm/μg, and when hybridization was efficient, giving a low background. Thus, these transcripts were analysed to determine their length, homology to specific intron probes, and to determine which were associated with the cellular translation machinery.

Transcript Size

The sizes of these transcripts were determined by comparing their migration to that of ribosomal RNA standards from *Saccharomyces* and *Drosophila* poly(A)$^-$ RNA samples. Figure 18 shows an ethidium bromide stained gel track containing these rRNA standards. A standard curve produced comparing the rRNA migration verses the length in kilobases is demonstrated in Figure 19. The length of the *Drosophila* rRNA standards were taken from Lis *et al* (1980) and the yeast rRNA sizes are taken from Lewin (1980) Table 27.1. Table 3 summarizes the size of each transcript as found in

Figure 18: H:V Northern gel rRNA size standards. Two micrograms of a mixture of yeast and *Drosophila* ribosomal RNA were electrophoresed in the flanking slots of most northern gels. After the electrophoresis was complete these slots were cut from the gel and visualized by ethidium bromide staining as described in the Materials and Methods. The positions of each rRNA transcript was transfered to the blot prior to baking and noted after autoradiography. The sizes of the *Drosophila* rRNAs were taken from Lis *et al* (1981). The *Drosophila* rRNAs correspond to the 2.32kb and the 1.9kb bands for the 28Sa and 28Sb molecules respectively and the 18S rRNA molecule is 2.16kb in length. The yeast rRNA 28S and 18S rRNA molecules are 3.75kb and 2.0kb respectively.

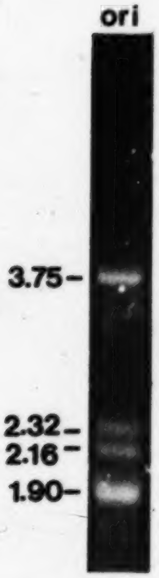

ori
3.75-
2.32-
2.16-
1.90-

Figure 19: Transcript length standard curve.

A typical standard curve produced by plotting the Log_{10} transcript length versus the distance migrated is shown here. The positions of the rRNA standards were visualized by ethidium bromide staining as described in the Materials and Methods. This was transfered to the gel blot prior to baking and measured after autoradiography.

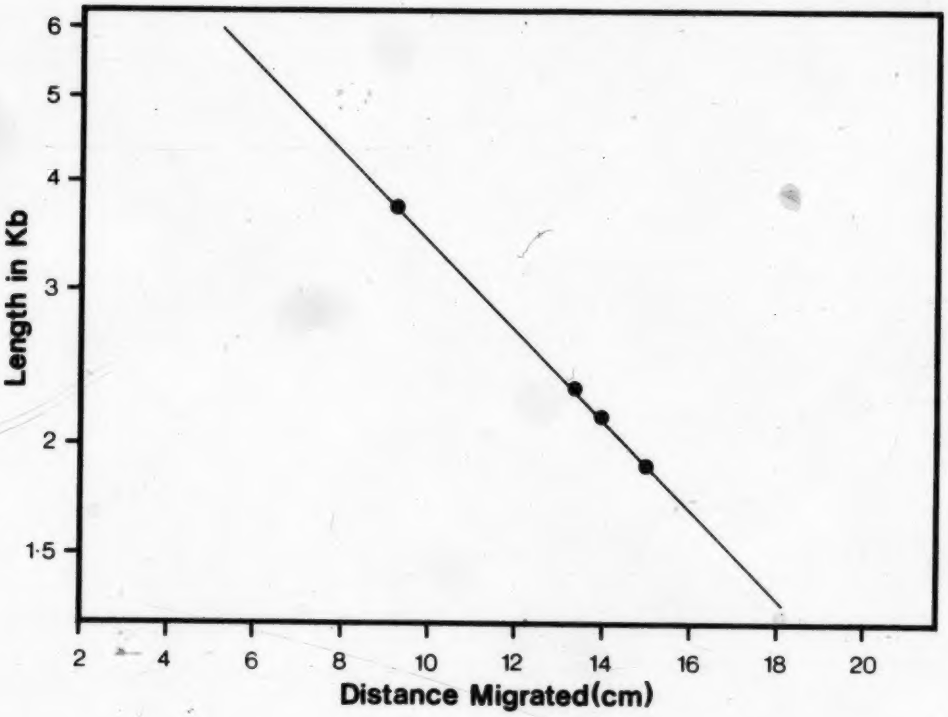

Length in Kb
6
5
4
3
2
1·5
2
4
6
8
10
12
14
16
18
20
Distance Migrated(cm)

Table 3: DDC transcript sizes.

The sizes of the DDC transcripts during early and late embryogenesis and larval samples were estimated from standard curves similar to that displayed in Figure 19. The sizes are the average of two estimations from two separate gels The sizes of the faint bands between the 3.25kb and the 4.5kb transcript in the 14 hr RNA sample were estimated using the sizes of the DDC transcripts.

TABLE 3

DDC TRANSCRIPT LENGTH

DEVELOPMENTAL TIME		
2 HR	14 HR	LARVAL
(2.0)	2.0	2.0
¤	2.15	2.15
2.2	¤	¤
¤	2.3	¤
¤	2.8	(2.8)
(3.25)	3.25	3.25
¤	(3.5)	¤
¤	(3.8)	¤
¤	(4.1)	¤
4.5	4.5	4.5
5.2	¤	¤

KEY

¤ not observed

( ) faint bands

Length in Kb

larval and embryonic stages in Figure 15. It should be noted that some of these values were obtained by extrapolation from the standard curve. In these northern analyses (Figures 13 and 15) the larval and the 14hr major doublet differ slightly in the size of the largest band. This difference in transcript pattern was found to be an artifact caused by the addition of yeast rRNA to the larval poly(A) RNA sample. This was necessary as the larval RNA sample contained a very high concentration of mature DDC transcript/μg of RNA compared to a late embryonic poly(A) RNA sample. To remedy this, immature third instar larval samples were collected to ensure that the concentration of the mature DDC transcript was comparable to levels in late embryonic RNA samples. In these larval RNA samples (L1 and L2) the transcript pattern is virtually identical to that seen in the 14 hr embryo RNA sample (Figure 20).

The embryonic transcript seen during early embryogenesis was determined to be 2.2kb in length (Table 3). This difference in median size is noticeable when compared to the 14 hr 2.3kb transcript in adjacent gel slots (Figure 21). The qualitative differences between the 2.2kb transcript and the closely sized 2.3kb transcript is addressed in the analysis to follow.

Figure 20: Comparison of larval and 14 hr embryonic transcripts.

The poly(A) RNA from two wandering third instar larval samples was prepared as described in the Materials and Methods. Two micrograms of each sample were electrophoresed along with a 14 hr embryonic RNA sample (Figure 15) in a H:V 1.5% agarose northern gel, blotted, and probed with 4.8kb *EcoR1* DDC DNA fragment as described in the Materials and Methods. The two larval RNA samples are designated L1 and L2. A and B represent 1 and 4 day autoradiographic exposure at -70°C respectively.

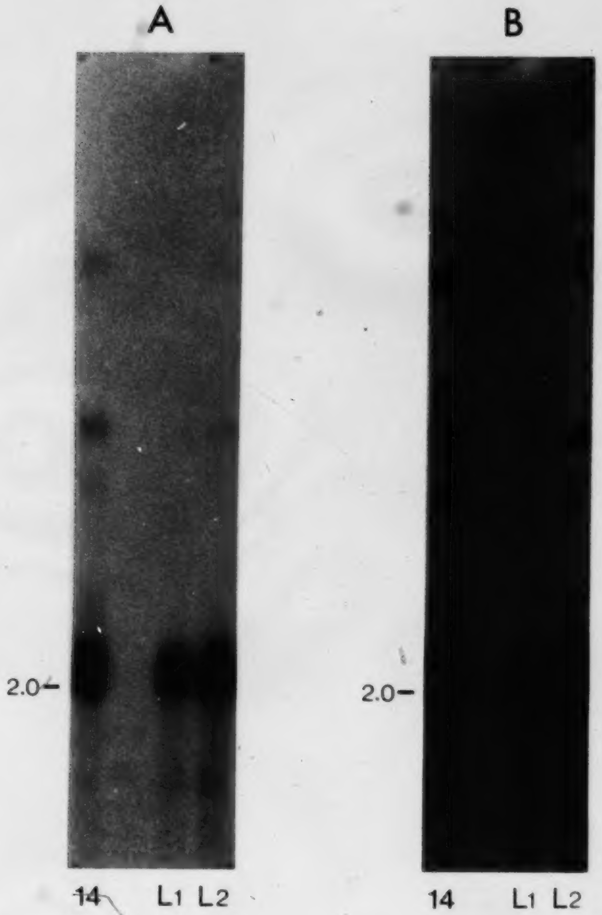

A
B
2.0
2.0
14
L1
L2
14
L1
L2

Figure 21: Comparison of 2 hr and 14 hr DDC transcripts. Two micrograms of total cellular poly(A) mRNA from both 2 and 14 hr RNA samples were electrophoresed in a H:V 1.5% agarose northern gel, blotted, and probed with the 4.8kb *EcoR1* DDC DNA fragment as in the Materials and Methods. The size indicated is the position of the 2.0kb 14 hr DDC transcript (see Table 3).

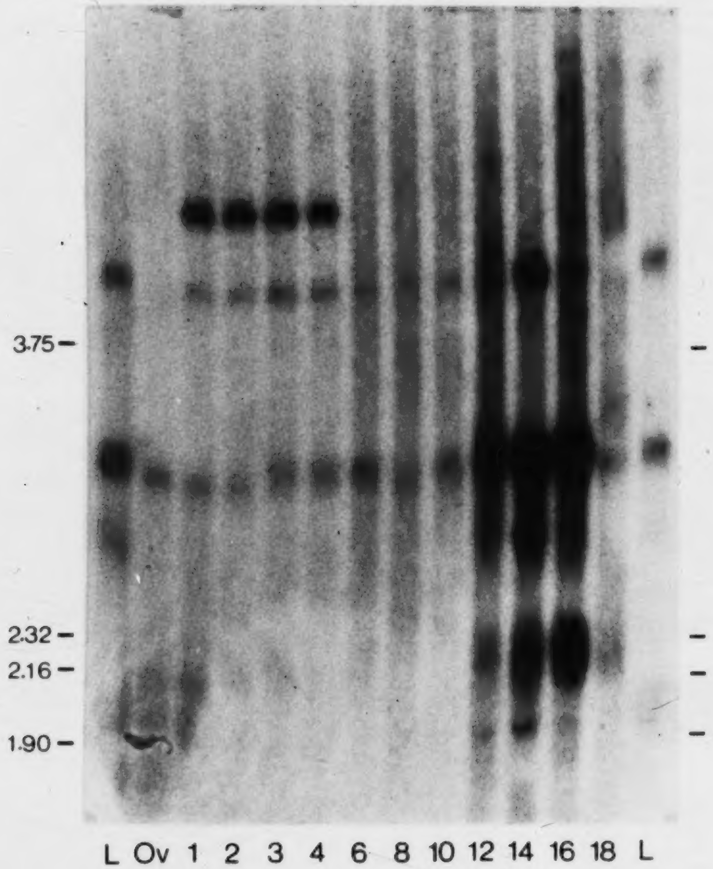
2.0–
2 14 14 2

Intron Probes

In an attempt to define the functional aspects of the different transcripts seen during embryogenesis the 4.8kb *EcoR1* DNA fragment was subcloned into smaller restriction fragments (see Figure 8) Both the 0.6kb *EcoR1-BamH1* and the 0.3kb *Xho1-HindIII* DNA fragments, which represent partial sequences from the 5' and the 3' introns respectively, as determined from DNA sequence analysis (R.B. Hodgetts, personal communication) and the data presented in Scholnick *et al* (1983), were used as probes of northern gel blots. However, due to the incomplete nature of the results with the 0.3kb *Xho1-HindIII* probe only the autoradiogram from a gel blot probed with the 0.6kb *EcoR1-BamH1* is presented. Northern gel blots were first hybridized to the 4.8kb DNA fragment to determine the transcript pattern and then blots were stripped of all radioactive probe with a high stringency wash and rehybridized to the subcloned DNA fragments. Figure 22 displays an autoradiogram produced with the 0.6kb *EcoR1-BamH1* subcloned fragment as probe. The absence of the major transcript response in both early (1 and 2) and late embryos (14 and 16) and in the larval sample (L) is immediately noticeable. All other transcripts are visible when the transcript pattern is compared to that found with the 4.8kb *EcoR1* probe (Figure 15). This result is consistent with the idea that the major transcripts are the mature mRNA species and the other mRNA transcripts represent precursors of this mature transcript. The presence of this

Figure 22: Northern gel analysis with the 0.6kb *EcoR1-BamH1* DDC intron probe.

The northern gel blot from Figure 15 was stripped of the 4.8kb *EcoR1* DDC probe as described in the Materials and Methods. The blot was then re-exposed at -70°C for 4 days which indicated that all discernable hybridization response of the 4.8kb *EcoR1* probe to the precursor transcripts was removed. A small amount of signal still remained hybridized to the most prevalent larval and 14 hr transcripts. After prehybridization the blot was probed with nick translated 0.6kb *EcoR1-BamH1* DDC DNA fragment. The larval RNA samples are designated, L, the ovary RNA sample, Ov, and the mean developmental age of the embryo RNA samples is indicated. The autoradiogram represents a 48 hr exposure at -70°C.


3.75
2.32
2.16
1.90
L Ov 1 2 3 4 6 8 10 12 14 16 18 L

0.6kb *EcoR1-BamH1* sequence in all these precursor transcripts suggest that this intron may be removed from the initial transcript in numerous pieces.

Subcellular Localization of RNA Transcripts

The complexity of the transcript patterns in both the early and the late embryo RNA samples prompted further investigation into their subcellular localization. Two and 14 hr embryo samples were collected and processed as described in the Materials and Methods to recover cytoplasmic and nuclear RNA from each. The resultant polyadenylated RNA samples were analysed on northern gel blots. The results for the 2 hr sample seen in Figure 23 show that most of the transcript at this stage is found in the cytoplasmic fraction of the RNA sample (2c) with only a minor amount of the total transcript visualized in the 2 hr nuclear RNA fraction (2n). This could be due to the crude nature of the nuclear RNA preparation.

In neither 14 hr sample was poly(A) was recovered from the nuclear RNA preparations. However, in Figure 24 the two cytoplasmic RNA samples both produce the major doublet and minor amounts of all larger transcripts. In both cases it seemed that all transcripts are associated with the cytoplasmic RNA preparations. As transcript maturation is supposed to occur exclusively in the nucleus (Darnell, 1982; Nevins, 1983), the reason for this result was not clear. Lenk *et al* (1977) have shown that when the detergent NP-40

Figure 23: Subcellular localization of the 2 hr embryonic transcripts.

A 2 hr embryo sample was extracted to yield both nuclear (2n) and cytoplasmic (2c) poly(A) RNA which was electrophoresed in a H:V 1.35% northern gel, blotted, and probed with the 4.8kb *EcoR1* DDC DNA fragment. The size markers are as in Figure 14. The gel blot was exposed for 2 days at -70°C.

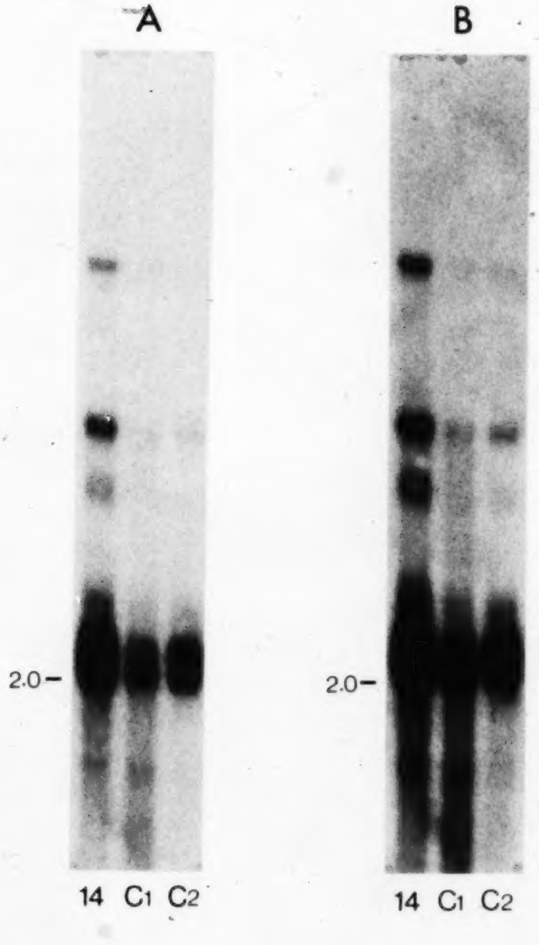
2.0–
14 2n 2c

Figure 24: Subcellular localization of the 14 hr embryonic transcript.

Two samples of 14 hr embryonic cytoplasmic poly(A) RNA (C1, C2) were prepared as described in the Materials and Methods. Two micrograms of each cytoplasmic RNA sample along with 2 μg of 14 hr poly(A) RNA sample (Figure 15) were electrophoresed, on a H:V 1.5% agarose northern gel, blotted, and then probed with the 4.8kb *EcoR1* DDC DNA fragment The size indicated is that of the 2.0kb DDC transcript.

A) 1 day exposure.

B) 3 day exposure.


A
B
2.0
2.0
14
C1
C2
14
C1
C2

was present in an extraction buffer this caused the loss of the nuclear membrane and subsequent leakage from the unbroken nucleus. Perhaps the presence of the precursor transcripts in the 14 hr cytoplasmic RNA preparation is due to their leakage from the NP-40 extracted nuclei. To determine the transcripts that are responsible for the production of the DDC protein, a polysomal analysis was undertaken for both early and late embryonic samples.

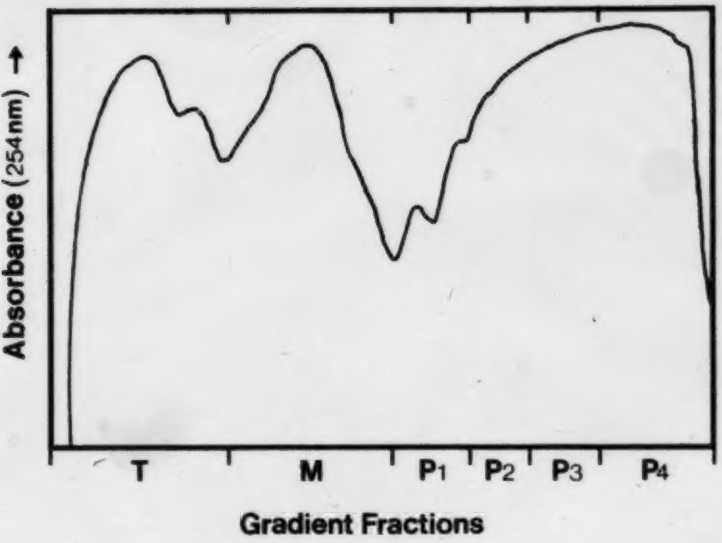
Polysome Analysis

Samples were homogenized using the non-ionic detergent NP-40, to solubilize membranes, and then size-fractionated using sucrose gradient centrifugation. Figure 25 displays a representative U.V.(254nm) absorbance profile from each stage produced during polysome gradient fractionation. These indicate that little if any degradation of the polysomal integrity has occurred. Polyadenylated RNA was recovered from each gradient fraction by phenol extraction and oligo-dT chromatography and then analysed using northern gel blots. Figure 26 shows the result from three early embryonic polysome gradients. The first two polysome gradients were produced from samples as described in the Materials and Methods and represent embryos 1 and 2 hr of age. In both gradients 1 and 2, the majority of the transcript is found in the top portion of the sucrose gradient representing messenger ribonucleoprotein (mRNP)-sized particles between 10-80S in fractions T and M respectively. In these first two

Figure 25: Polysome gradient fractionation absorbance profile.

The absorbance (254nm) profile of a 14 hr embryonic and a 2-4 hr embryonic polysomal gradients are displayed. The fractions collected are indicated on the bottom axis where T represents the top of the gradient, M, the monosome fraction, and P, the polysome fraction of the gradients.

14hr


Absorbance (254nm) →
T
M
P1
P2
P3
P4
Gradient Fractions

2hr

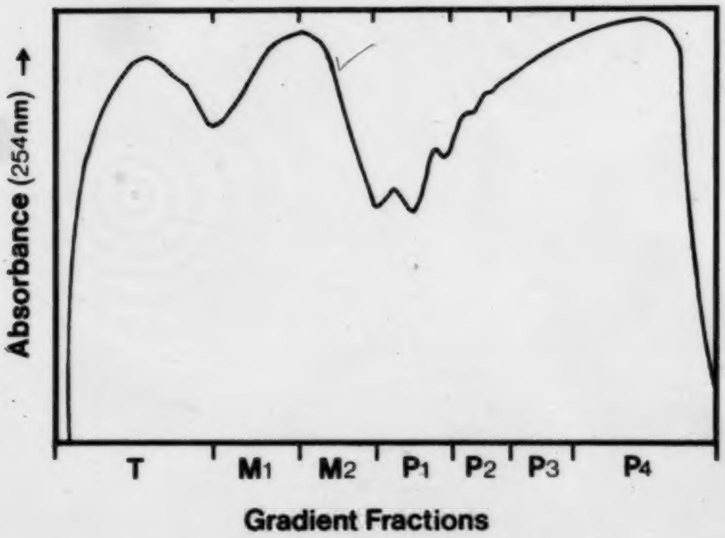

Absorbance (254nm) →
T
M1
M2
P1
P2
P3
P4
Gradient Fractions

Figure 26: Polysome analysis of early embryonic DDC transcripts.

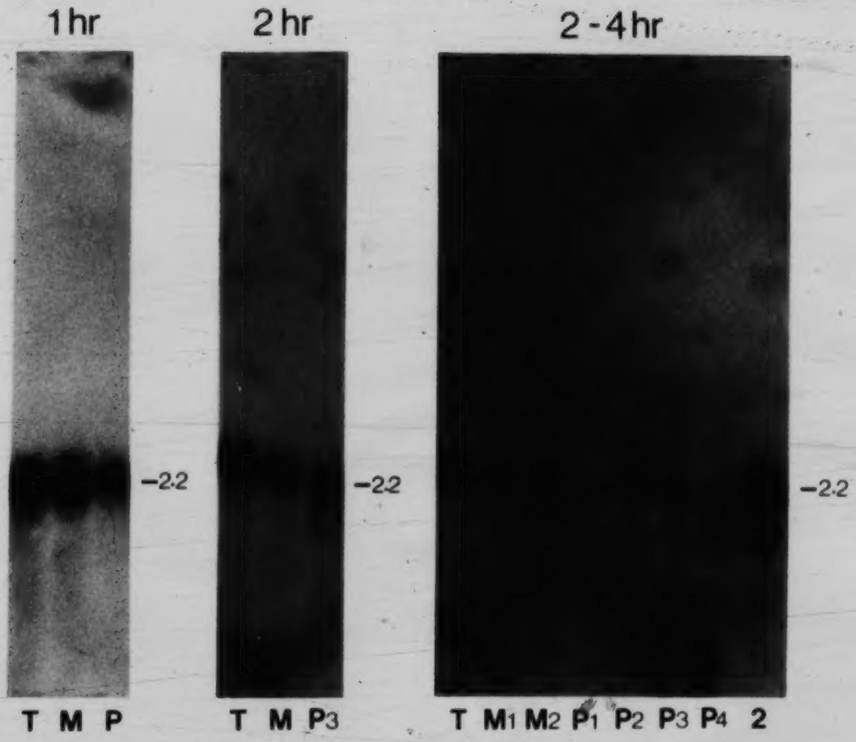
Three embryonic samples with mean developmental ages of 1, 2, and 2-3 hr were homogenized and fractionated using sucrose gradient centrifugation as described in the Materials and Methods.

A) 1 hr embryo sample: The 2 sucrose gradients were each fractionated into 3 fractions (T, M, P) and the poly(A) RNA isolated from each fraction. Two micrograms of the poly(A) RNA from each fraction were analysed using northern gel blots.

B) 2 hr embryo sample: The sucrose gradients were fractionated into 5 fractions (T, M, P1, P2, P3) but only those represented in the blot yielded poly(A) RNA.

C) 2-4hr embryo sample: The gradients were fractionated into 7 fractions (T, M1, M2, P1, P2, P3, P4) as shown in Figure 22 which all yielded poly(A) RNA. The slot designated 2 represents a 2 hr cytoplasmic poly(A) RNA sample.

Each gel sample represents 2 μg of poly(A) RNA electrophoresed in a H:V 1.5% agarose northern gel, blotted, and probed with 4.8kb *EcoR1* DDC DNA fragment.


1 hr
2 hr
2 - 4 hr
–2.2
–2.2
–2.2
T M P
T M P3
T M1 M2 P1 P2 P3 P4 2

samples only a minor fraction of the transcript is associated with ribosomes and migrates to the polysome portion of the gradient.

The third polysome gradient was produced using a sample collected for 4 hours at 20°C and then aged at 29°C for 1 hour. This was done to give a wider age distribution to the embryo sample in an attempt to include embryos between 2 and 3 hr of age. In comparison to the first two gradients, a smaller proportion of the total 2.2kb transcript found in this sample is seen in the prepolysomal part of the gradient, and more is found associated with the polysomal fractions (see 2-4 hr, fractions P1 through P4, Figure 26). This suggests that this early transcript is associated with the ribosomes and possibly translated. The last fraction of this gradient contains a minor amount of what appears to be 2.0kb mature transcript. This transcript is also seen in the polysome fraction P3 from gradient 2 (Figure 26) and in the 3 and 4 hr samples in figure 15B, C, where the 2.2kb transcript has completely disappeared. The fact that the 2.2kb transcript is prepolysomal until after 2 hr and the 3 hr sample only contains a small amount of 2.0kb transcript suggests that the 2.2kb transcript could be acting as a precursor to this 2.0kb RNA molecule. Interestingly, the DDC baseline activity increases sharply between 3 and 4 hr of embryonic development (Table 4). Thus, it seems that the early maternal DDC transcript is not translated until some time between 2 and 3 hr of development, at which time it

Table 4: DDC activity during early embryogenesis. Early embyro samples were collected for 1 hr and then aged (at 25°C) from the midpoint of collection period. The samples were extracted and assayed for DDC activity as described by Clark *et al* (1978) except the sample volume assayed was increased to 200µl and the amount of dopa added to the reaction was doubled. All assays were kindly done by Charlotte Spencer.

TABLE 4

DDC ACTIVITY IN EARLY EMBRYOGENESIS

	DDC ACTIVITY (UNITS)	
SAMPLE AGE	EXPT[1]	EXPT[2]
2.0 HR	0.028	N.D.
2.5 HR	0.033	0.039
3.0 HR	0.029	0.033
3.5 HR	0.059	0.033
4.0 HR	0.069	0.031
4.5 HR	0.065	0.077
5.0 HR	N.D.	0.098

1 unit = 1 nanomole L-dopa decarboxylated/hr/mg tissue

produces a sharp increase in the basal DDC activity level.

Polysome analysis was also carried out on 14hr embryo samples. A representative northern blot is displayed in Figure 27. In each exposure the major doublet is predominantly in the last gradient fraction (P4). This sedimentation behaviour indicates that both the 2.0kb and the 2.15kb transcripts may be associated with ribosomes. Interestingly, the 2.3kb transcript is also prominent in this gradient fraction (P4). This RNA molecule still contains intron sequence as shown by its hybridization to the 0.6kb *EcoR1-BamH1* probe (Figure 22). Minor amounts of the 2.3kb and 2.15kb transcripts are also seen in all polysome fractions (P1 through P4) and in the monosome fraction (M). The 2.0kb transcript, found only in the last two gradient fractions (P3 and P4), may be produced from another transcript by a processing event after or during translation.

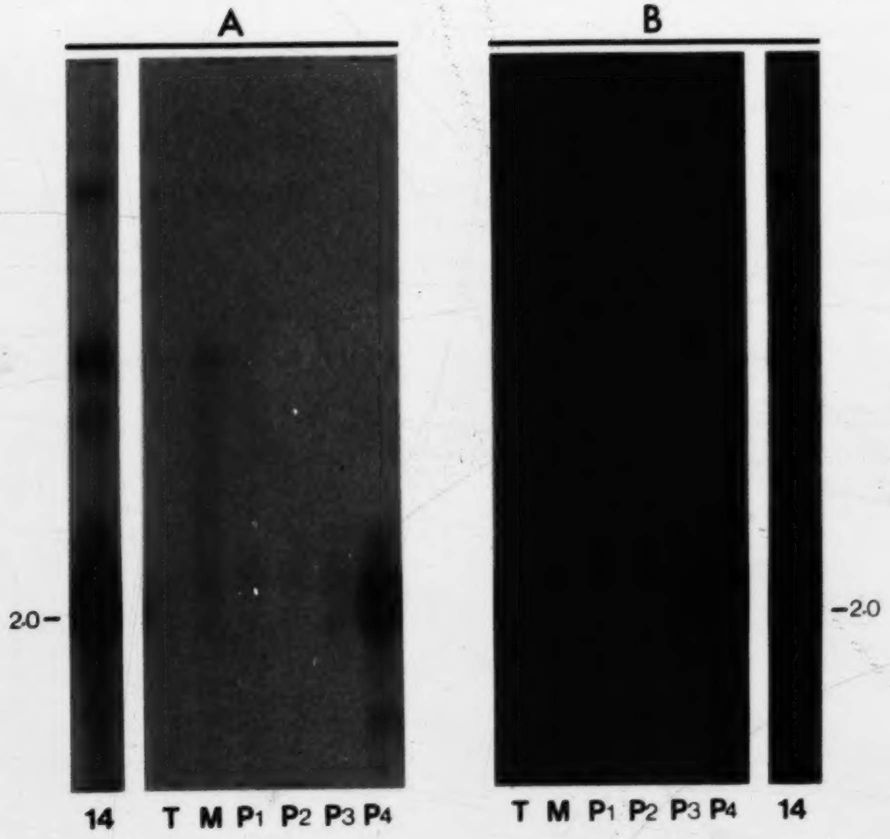
Also notable is the presence of precursors in the second gradient fraction (M) which contains single 80S ribosomes mRNP particles. All precursor transcripts are visible in Figure 27B. The migration of these transcripts to the 80S size fraction of the gradient suggests that the RNA is in the form of messenger ribonucleoprotein complexes. Their narrow size distribution is contrasted by the large variation in the size of the 2.2kb prepolysomal RNA transcripts seen in the early embryonic polysome gradients (Figure 26). The significance of this is not understood.

Figure 27: Polysome analysis of 14 hr embryonic DDC transcripts.

A three hundred mg sample of live 14 hr embryos was homogenized and fractionated using sucrose gradient centrifugation as described in the Materials and Methods and the gradients were collected as shown in Figure 25 (14 hr). Two micrograms of poly(A) RNA from each gradient fraction were analysed in a H:V 1.5% agarose northern gel, blotted, and probed with the 4.8kb *EcoR1* DDC DNA fragment. The size indicated represents the position of the 2.0kb DDC transcript.

A) 2 day exposure.

B) 4 day exposure.


A
B
2.0
2.0
14
T M P1 P2 P3 P4
T M P1 P2 P3 P4
14

Thus, from this analysis we see that the 2.15kb and the 2.0kb DDC transcripts are associated with multiple ribosomes indicating these transcripts are translated. The 2.3kb transcript is also found in the high molecular weight fraction of the sucrose gradient, however, due to the presence of intron sequences in this transcript, further investigation is necessary to determine if it is associated with cellular translation machinery. The transcripts larger than 2.3kb are exclusively found in the 80S fraction of the sucrose gradient indicating they are not associated with multiple ribosomes and are probably present in these preparations due to the NP-40 in the extraction buffer.

In Vitro TRANSLATION OF EMBRYONIC mRNA

The northern gel blot analysis shown earlier (Figure 20) revealed that the mRNA transcript profile in both embryos and prepupal stages were similar. An analysis of the proteins produced by *in vitro* translation of these mRNAs was undertaken in an attempt to compare the DDC proteins from each stage. Polyadenylated mRNA from each time point was translated using a rabbit reticulocyte translation system. Figure 28 displays a fluorogram of a SDS polyacrylamide gel analysis of the radioactive proteins produced by translation. This shows that some changes occur in the spectrum of mRNAs present from the end of oogenesis (Ov) through to the end of embryogenesis (2-16), with the larval RNA sample (L) producing a distinct protein profile.

Figure 28: *In Vitro* translation products from embyonic RNA. The *in vitro* translation products from each embryonic RNA sample (Figure 12) were analysed on a SDS 10% polyacrylamide gel. The gel was fixed in 10% acetic acid and then prepared for fluorography as described in the Materials and Methods. Each slot contains approximately 8 x 10^5 cpm acid insoluble ^{35}S-methionine incorporated into protein. The larval RNA samples are designated, L, the ovary RNA sample, Ov, and the mean developmental time of each embryo RNA sample is indicated. The molecular weights indicated are derived from the BioRad SDS-PAGE Low molecular weight standards,

92.5 kd: Phosphorylase B
66.2 kd: BSA
45.0 kd: Ovalbumin
21.5 kd: Soy Bean Trypsin Inhibitor
14.4 kd: Lysozyme

The fluorogram was exposed for 12 hrs at -40°C.

Total

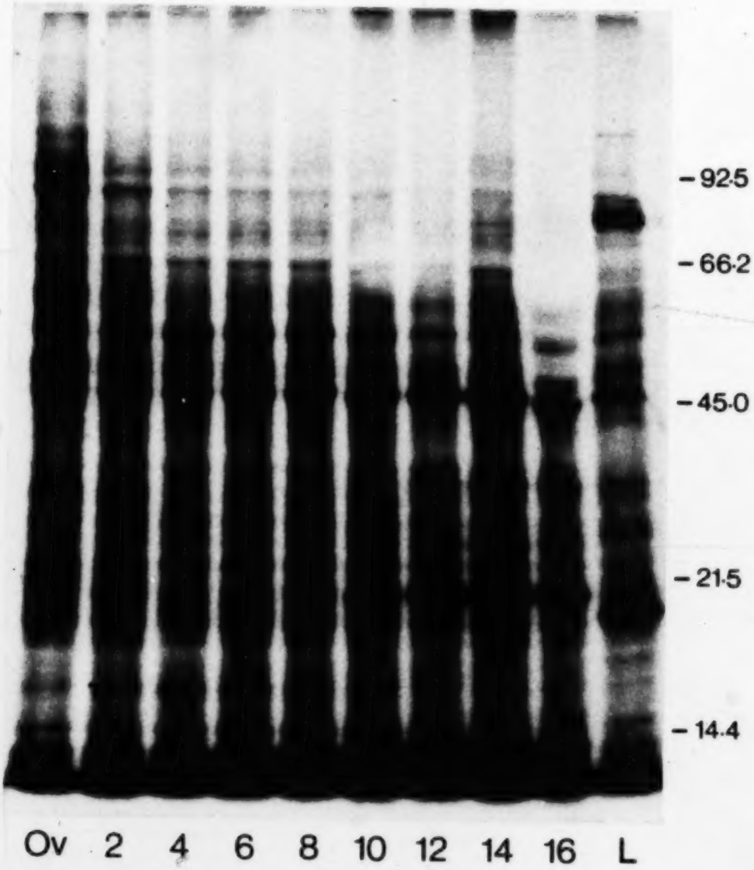


Figure 29: Immunoprecipitation of embryonic DDC. The *in vitro* translation products from the cell free translation of each embyronic RNA sample (Figure 28) were immunoprecipitated with anti-DDC IgG as described in the Materials and Methods. The embryonic (2-16 hr) immunoprecipitates were analysed along with larval samples (L) using a SDS 10% polyacrylamide gel. The gel was fixed with 10% acetic acid and prepared for fluorography as described in the Material and Methods. The arrow indicates the DDC protein subunit of 54 kd. The molecular weight indicated represents the position of the major mRNA dependent lysate translation product. The fluorogram was exposed for 35 days at -40°C.

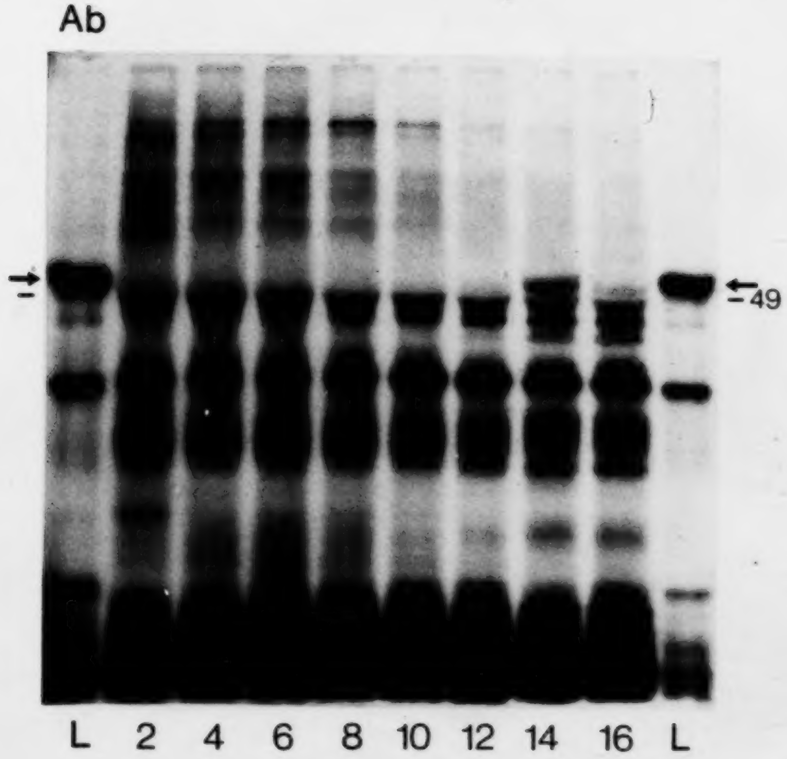

Ab
49
L
2
4
6
8
10
12
14
16
L

Figure 29 shows the fluorogram of a SDS polyacrylamide gel analysis of the proteins immunoprecipitated with anti-DDC IgG from *in vitro* translation products from each embryonic RNA sample (2-16). The numerous proteins seen in each sample is due to non-specific adsorption of the IgG and the *S. aureus* ghosts. This type of non-specific adsorption can be controlled in samples containing large amounts of antigen by pre-adsorption with preimmune IgG prior to antibody immunoprecipitation (Figure 10). However variations in batches of antibody and *S. aureus* ghosts still produced some uncontrollable non-specific adsorption even after preadsorption with control IgG, as noticeable in the larval samples (L) in Figure 29. The utilization of protein-A:Sepharose 4CL-B (Pharmacia) as solid phase immunoadsorbant did reduce some of the binding problems but the major portion of the nonspecific binding seemed to be attributable to antibody batch variation.

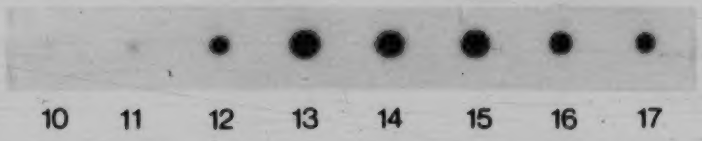
Proteins comparable in size to the larval DDC subunit (indicated by arrow in Figure 29) are also seen in the 14 and 16 hr samples suggesting that the DDC protein produced during embryogenesis is similar in size to the larval protein. However, due to the large amounts of anti-DDC IgG used in each immunoprecipitation each slot is overloaded with IgG producing a major protein band of IgG heavy chain peptide migrating to 50,000 daltons in size on a SDS-PAG. This distorts the migration of the proteins of similar size in each slot, thus no conclusive statements can be made about the nature of the translation products of these DDC transcripts. Perhaps this problem could be alleviated by denaturing the immunoprecipitates in the absence of β-mercaptoethanol. In the absense of this reducing agent the IgG molecule should not breakdown into its component light and heavy peptides, causing it to migrate to a different position in the gel, not interfering with the migration of the DDC proteins.

Due to these poor results an attempt was made to obtain an immunoprecipitate, in which the non-specific binding was minimal, through partial purification of DDC mRNA.

Polyadenylated mRNA from early and late embryo samples was size-fractionated using sucrose gradient centrifugation. The location of the transcripts homologous to the 4.8kb probe was determined by RNA dot blot hybridization analysis of the gradient fractions (Figure 30). The fractions containing the most DDC transcript were then analysed using northern gel blots to determine the spectrum of transcripts found in each. Figure 31 and 32 show the results from the 2 hr and 14 hr gradient analysis respectively. Attempts to translate and immunoprecipitate the embryonic DDC from fractions containing the 2.0kb and the 2.15kb RNA molecules failed to eliminate the problem of nonspecificity during immunoprecipitation. It seems that it is virtually impossible to obtain clean immunoprecipitates unless one is dealing with a reasonably concentrated antigen. Therefore further DDC transcript purification was attempted using

Figure 30: RNA Sucrose gradient fraction dot blots. Three hundred micrograms of poly(A) RNA from 2 and 14 hr embryo samples were size fractionated using SDS sucrose gradient centrifugation as described in the Materials and Methods. The sucrose gradient fractions containing DDC transcripts were determined by dotting 0.75μg of poly(A) RNA from each gradient fraction to nitrocellulose and probing with the 4.8kb *EcoR1* DDC DNA fragment as described in the Materials and Methods. In both the 2 hr and the 14 hr gradients fractions 10-17 are shown. The autoradiogram was exposed for 24 hours at -70°C.

2 hr



14 hr

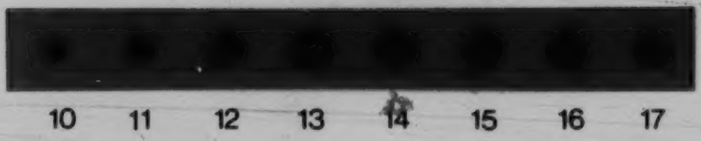


Figure 31: H:V Northern analysis of 2 hr RNA gradient fractions.

One half a microgram of poly(A) RNA from gradient fractions 10 through 17 was electrophoresed in a H:V 1.5% agarose northern gel, blotted, and probed with the 4.8kb *EcoR1* DDC DNA fragment. The size indicated is that of the 2.2kb DDC transcript (see Table 3). The autoradiogram was exposed for 4 days at -70°C.

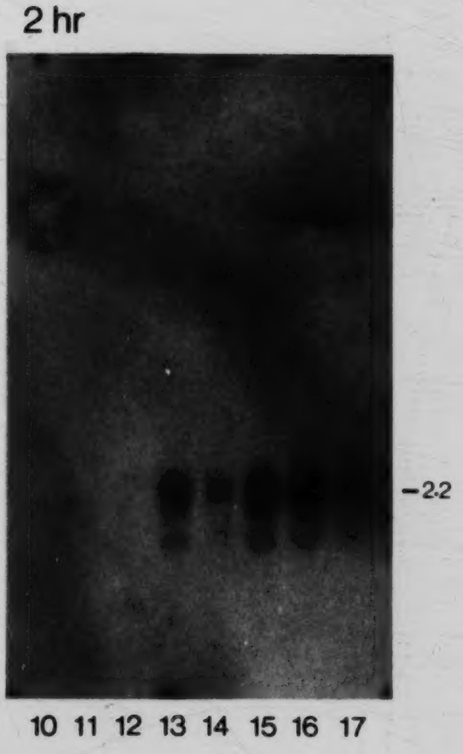
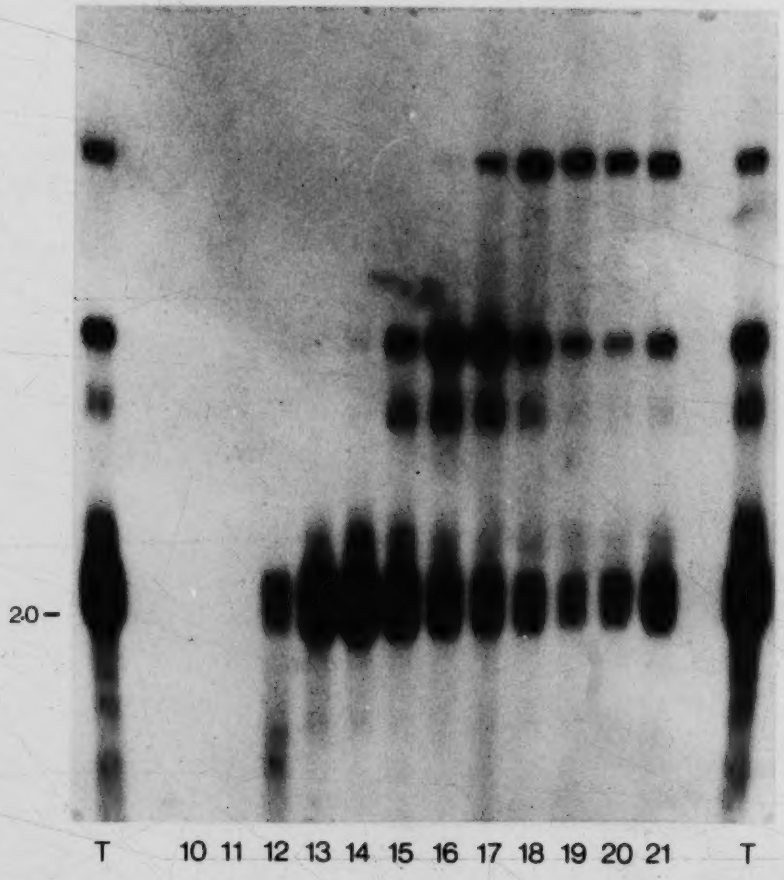

2 hr
-2.2
10
11
12
13
14
15
16
17

Figure 32: H:V Northern analysis of 14 RNA gradient fractions.

One half a microgram of poly(A) RNA from gradient fraction 10 thru 21 was electrophoresed on a H:V 1.5% agarose northern gel, blotted, and then probed with the 4.8kb *EcoR1* DDC DNA fragment. The size indicated is the position of the 2.0kb DDC transcript. The autoradiogram was exposed for 2 days at -70°C.

14 hr



hybridization selection of molecules homologous to the 4.8kb DNA fragment (Riccardi *et al*, 1979; Parnes *et al*, 1981). This approach worked well in purifying translatable DDC transcripts from larval RNA samples, however, due to the limited supply of 14 hr embryonic RNA, the recovery of translatable embryonic DDC transcripts was never successfully accomplished. Again it seems the low concentration of the DDC transcripts in the 14 hr embryonic RNA samples makes this technique difficult to use. In conclusion, I feel that both immunoprecipitation and mRNA hybridization selection are techniques that are difficult to apply if one is studying an antigen or RNA species that is in low abundance.

IV. DISCUSSION

The work presented here represents a continuation in the analysis of the factors influencing DDC expression throughout development in *Drosophila melanogaster*. All initial studies in this laboratory have investigated the expression of DDC during pupariation and its relationship to the moulting hormone titer peak also at this time (see Figure 1). Kraminsky *et al* (1980) showed that the increase in DDC activity at this stage was due to an ecdysone stimulated increase of translatable mRNA. Recently W.C.Clark (personal communication) has shown that this response seems to be independent of protein synthesis, suggesting that the induction of translatable mRNA is at the level of gene transcription. This study focuses on DDC expression during embryogenesis and its relationship to the ecdysteroid peak at that time.

DDC EXPRESSION DURING EMBRYOGENESIS

The DDC activity found during embryogenesis is shown in Figure 2. These data show that the appearance of DDC activity is limited to the end of embryogenesis, just prior to first instar larvae hatching. This temporal pattern correlates well with the appearance of the embryonic cuticle at 12 hr of development (25°C) reported by Hillman and Lesnik (1970). This suggests that the DDC activity during embryogenesis may be involved in sclerotization of this cuticle.

Of primary interest in this study is the relationship of the embryonic ecdysteroid titer peak, also seen in Figure 2, to the appearance of the DDC activity at this stage. The apparent stimulatory effect of 20-OH ecdysone on DDC expression during pupariation raised the question whether the moulting hormone also stimulates or controls the production of DDC activity during embryogenesis. However, the temporal relationship between the ecdysteroid peak and the DDC activity during embryogenesis differs from that seen at pupariation. Hodgetts *et al* (1977) showed that during pupariation both ecdysteroid titer and DDC activity rise almost concurrently. Figure 2 shows that during embryogenesis no substantial DDC activity is found until the hormone titer has dropped back to baseline levels. The temporal differences in DDC expression between pupariation and embryogenesis are similar to the early and late polytene chromosome puffing responses to 20-OH ecdysone stimulation in the salivary glands of *Drosophila melanogaster* (Ashburner, 1974), respectively. The early puffing represents transcription induced within minutes after 20-OH ecdysone application. This is thought to be a primary response to the interaction of the hormone:receptor complex at the DNA level as it is refractory to the effects of protein synthesis inhibitors. Conversely, the late puff induction is controlled by the hormone but occurs hours after the initial hormone application and requires a number of events to initiate puffing as demonstrated by the sensitivity of their induction to protein synthesis inhibitors. This system, exhibiting two temporal patterns in response to ecdysone stimulation, corresponds to different sets of molecular events at the level of gene regulation. The control of DDC expression may represent a similar system.

The difference in temporal relationship between the moulting hormone titer peak and the onset of DDC activity during embryogenesis, when compared to that found during pupariation, suggests that regulation of DDC expression with respect to the moulting hormone is similar to the late puffing scenario. Alternatively, the temporal lag could be a manifested for some other reason. The possibility that the DDC activity at this stage is produced by a different structural gene from that responsible for activity found during pupariation was investigated.

EMBRYONIC DDC IDENTITY

Recently, Batterham *et al* (1983) have shown that the alcohol dehydrogenase activity found in *Drosophila mojavensis* is produced from two distinct ADH structural genes. These two ADH genes are expressed in different temporal and tissue-specific patterns during the ontogeny of this species. The authors believe that this situation was produced by a gene duplication event giving rise to similar ADH genes whose control over expression have diverged to the present situation.

The DDC enzyme found during embryogenesis in *Drosophila melanogaster* was first compared to that seen at pupariation using antibody raised against DDC purified from late third instar larvae. The interaction of the embryonic DDC with this antibody in both the immunotitration (Table 1) and the double immunodiffusion (Figure 6) experiments suggest that the enzymes at these two stages are immunologically similar.

To rule out the possibility that two structurally similar genes exist for DDC activity in *Drosophila melanogaster* the analysis was extended to determine the effect of the mutation *l(2)Ddc*^{ts2} on DDC activity at both stages. The temperature sensitive lethal mutation *l(2)Ddc*^{ts2}, isolated by Wright *et al* (1981), affects DDC activity levels in both white prepupae and newly eclosed adults reducing it to about 2% of wild-type levels. The DDC activity found in the mutant strain, at these stages, is more thermolabile than the wild-type enzyme when crude extracts are incubated at 56°C prior to assay (T.R.F.Wright, personal communication). This suggests that the lesion resides in the structural gene. This mutation also proved to affect DDC activity levels during embryogenesis (Table 2) and was shown to map to the location of the DDC structural gene defined by Wright *et al* (1976)(see results). These results along with the Southern blot analysis of Hirsh and Davidson (1981) and Estelle (personal communication) convincingly show that a single DDC gene exists in *Drosophila melanogaster*.

The previous result led us to investigate if a different type of embryonic ecdysteroid may be responsible for the temporal lag prior to DDC expression. Previous observations using RIA antibody binding showed that many forms of ecdysterone are detectable in this manner (Chang and O'Connor,1979). Thus the nature of the ecdysteroids present in the embryonic RIA peak was investigated.

EMBRYONIC ECDYSTEROID ANALYSIS

In *Manduca sexta* it has been reported that the embryonic ecdysteroid consists of primarily 26-OH ecdysone while at other stages of ontogeny of this insect the primary ecdysteroid is 20-OH ecdysone (Kaplanis *et al*, 1976). Investigation into the type of ecdysteroids in the embryonic titer peak showed that a large amount of RIA positive material was found to comigrate with authentic ecdysone and 20-OH ecdysone standards when analysed with TLC (Figure 5a). When re-analysed with HPLC these TLC zones contained compounds which co-chromatographed again with authentic ecdysone and 20-OH ecdysone in a molecular ratio of approximately 1:2 respectively (Figures 5b,c).

Thus in *Drosophila melanogaster* we find that the predominant embryonic ecdysteroid is 20-OH ecdysone, similar to the situation during pupariation (Hodgetts *et al*, 1977). Therefore the temporal lag between the embryonic ecdysteroid peak and the onset of DDC activity does not seem to be caused by a different embryonic ecdysteroid species.

Also noticeable in Figure 5a is a substantial peak of RIA positive material migrating at about 10cm on the TLC plate. Compounds with similar TLC Rf's have been seen in *Locusta* embryos and identified as both deoxyecdysones and dehyroxyecdysones (Lagueux *et al*, 1979). These compounds are thought to represent precursors and breakdown products of biologically active ecdysteroids respectively. The embryonic ecdysteroids in *Drosophila melanogaster* are presumably derived from maternally contributed precursors of the deoxyecdysone type.

Absent in these *Drosophila* embryonic ecdysteroids are the highly polar ecdysteroid conjugates found in the embryos of both *Locusta migratoria* (Lagueux *et al*, 1979) and *Shistocera gregaria* (Gande and Morgan, 1979). The conjugation of biologically active ecdysteroids is believed to be a rapid method of inactivation and the first step in the recycling or degradative pathway. The absence of these highly polar conjugates from the *Drosophila* sample might be due to an inability to detect these types of molecules with the antisera used, although this antisera detected ecdysteroid conjugates in 9 day old *Locusta* embryo extracts (B.Sage, personal communication). More likely, *Drosophila* embryos may inactivate the ecdysteroids in an alternate manner obviating the production of these highly polar conjugates. More investigation is needed for this to be determined however.

The significance of this embryonic ecdysteroid peak in *Drosophila* is enigmatic. The titer peak does however occur just prior to the formation of the embryonic cuticle at 12 hours of development at 25°C (Hillman and Lesnik, 1970). Lagueux *et al* (1979) have shown that each of the four embryonic ecdysteroid titer peaks in *Locusta migratoria* correlates temporally with the formation of an embryonic cuticle. This suggests that in *Locusta* these embryonic ecdysteroids are involved in the regulation of embryonic cuticle synthesis. The significance of the *Drosophila* ecdysteroid peak could possibly be resolved if mutations affecting its appearance could be identified. Wright (1970) has reviewed many of the mutants in *Drosophila melanogaster* whose effect is manifested during embryogenesis. Perhaps analysis of those affecting development around the time of the ecdysteroid peak may identify lesions involved in ecdysteroid production at this stage. This type of mutation would be useful in determining the effects that the embryonic ecdysteroids have on gene regulation.

The mutation *l(3)ecdysoneless-1*^{ts} isolated and described by Garen *et al* (1977) eliminates the puparial ecdysteroid peak when raised at the restrictive temperature (29°C) during third instar larval growth. This mutation's effect on the embryonic ecdysteroid peak and DDC activity was investigated in the hope that it might prove a useful tool in analysing the relationship between them.

THE EFFECT OF *l(3)ecdysoneless-1*^{ts} DURING EMBRYOGENESIS

Kraminsky *et al* (1980) have shown that *l(3)ecdysoneless-1*^{ts} larvae raised at 29°C in the absence of dietary 20-OH ecdysone fail to produce an increase in DDC activity during late third instar. This mutant has proved to be a valuable tool in the analysis of DDC expression during pupariation. To analyse the relationship between DDC expression and the ecdysteroid peak during embryogenesis the utility of the *l(3)ecdysoneless-1*^{ts} mutation as a tool to study their interrelationships was investigated. Figures 3 and 4 show however that at both the permissive and the restrictive temperatures the *ecd*^{1} mutant strain was indistinguishable from the wild-type for both ecdysteroid and DDC appearance. Thus this mutation proved to be of no use for the analysis of DDC expression and its relationship to the ecdysteroids present during embryogenesis. Redfern and Bownes(1983) have extensively analysed *l(3)ecdysoneless-1*^{ts} and believe that its pleiotropic phenotype, including the affect on ecdysteroid accumulation during late third instar, is produced because the lesion is a temperature-sensitive cell-autonomous lethal mutation which affects specific mitotically active cells. Thus, not directly involved in ecdysteroid biosynthesis, this gene when mutant, only indirectly affects ecdysteroid accumulation at pupariation by its lethality exerted on cells involved in the hormone production. This mutation does not affect ecdysteroid accumulation during embryogenesis

(Figures 3 and 4) because its lethal effects are not significant to the hormone's production at this stage.

DDC TRANSCRIPT EXPRESSION DURING EMBRYOGENESIS

From the previous analyses we know that both embryonic ecdysteroids and DDC enzyme molecules are of identical types when compared to those found during pupariation. Therefore the temporal lag between the embryonic ecdysteroid peak and the onset of DDC activity can be considered to be manifested by an interaction between the two, similar to the late puffing response mentioned above. Alternatively, the interaction between the embryonic ecdysteroids and the DDC gene may be similar to the situation at pupariation, causing the immediate production of DDC mRNA that is stored in a non-translatable form until it is used later during embryogenesis. It has been shown that transcription of a gene and subsequent translation to give rise to overt expression can be temporally separated by translational control over the transcript produced (see Kastern *et al*, 1982; Ballinger and Pardue, 1983; and Rosenthal *et al*, 1983). This was investigated using Northern gel blotting methods.

Two sets of *Canton-S* embryo samples were collected to represent time points throughout embryogenesis at 29°C and DDC transcript accumulation at each stage was analysed using Northern gel blots of the RNA extracted. Substantial accumulation of transcripts homologous to the 4.8kb *EcoR1*

DDC clone (Figure 8) are visualized at two periods during embryogenesis (Figures 12,13 and 15). A significant amount of DDC transcript is found in the RNA pools of pre-blastoderm embryos. This was unexpected as only low levels of DDC activity can be measured at this time.

Also noticeable from these Northern gel blots (Figures 13 and 15) is the accumulation of a variety of different sized RNAs which arise beginning at 12 hr of development just prior to the increase in DDC activity. Thus the embryonic ecdysteroid peak, occuring at 8 hr, seems to have no immediate effect on DDC transcript accumulation. This is clearly demonstrated in Figure 16. Interestingly, DDC transcript levels peak at 14 hr of development, two hours before the peak in DDC activity, then decrease precipitously to baseline levels by 18 hr. This indicates that the half-life of the transcripts at this time is relatively short. Thus it seems that the interaction between the embryonic ecdysteroid peak and the DDC gene is analogous to the late puffing response of the salivary gland polytene chromosomes to the moulting hormone as described by Walker and Ashburner (1981). This is in contrast with the situation that occurs during pupariation, where the DDC gene seems to respond immediately to ecdysteroid stimulation.

This situation could reflect alternate promoter usage at the two stages. During embryogenesis DDC transcription may be initiated from a promoter unresponsive to hormone stimulation, while during pupariation transcription may be controlled from a promoter element requiring hormone stimulation. Schibler *et al* (1983) have shown that the tissue specific expression of the mouse α-amylase gene in the liver and the parotid gland is directed from two distinct promoter elements each defining a different mRNA start site. In the parotid gland a more efficient promoter is used to give rise to a greater accumulation of gene transcript necessary in that tissue. Recently, Benyajati *et al* (1983) have also shown that the expression of the ADH gene in *Drosophila melanogaster* is regulated from two distinct promoters which are used to initiate transcription at two different stages of development.

In both cases mentioned, the mature transcripts produced from each promoter differed slightly from each other in size. However the DDC transcript patterns observed here, from both late embryonic and prepuparial stages, proved to be indistinguishable from each other in size (Figure 20). If multiple mRNA promotion sites were being used at these stages one might expect to detect size variation in the mature transcript as well as precursor molecules and this is clearly not the case for the DDC transcripts at these two stages. Although more sequence information on the transcripts at both stages is needed, from this analysis it seems likely that the RNAs at both times in development are produced from the same promoter initiation site.

The apparent dichotomy in DDC gene regulation may result from the uncovering of an ecdysteroid responsive enhancer type element similar to the glucocorticoid responsive enhancer described by Chandler *et al* (1983). Possibly, this ecdysteroid enhancer may not be available to signals during embryogenesis due to constraints over its expression imposed by the chromatin structure around the gene at that time. Burch and Weintraub (1983), have shown that constraints over gene expression are reflected by the absence of DNaseI hypersensitive sites which are normally seen both 5' and 3' to the gene just prior to expression. Thus the chromatin structure in and around the DDC gene should be investigated at both stages. These studies in conjunction with an analysis of the interaction of the hormone:receptor complex with the DDC gene at each stage may provide some insight into the regulation of DDC gene expression and perhaps a better understanding of developmentally significant regulatory mechanisms.

DDC TRANSCRIPT CHARACTERIZATION

The final part of this thesis was devoted to a characterization of the numerous embryonic DDC gene transcripts. In what follows, a model is put forth that attempts to explain the complexity in terms of the processing of a primary gene transcript to a fully mature translatable messenger RNA.

The transcripts hybridizing to the 4.8kb *EcoR1* DDC probe at both early and late embryogenesis produce a complex pattern consisting of multiple bands with various steady state levels of accumulation (Figures 15B,C). The DDC gene has been reported to contain two intervening sequences (introns) each about 1 kb in length (Beale, Morgan, Morse, and Hirsh as cited in Scholnick *et al*, 1983). Thus this mixture of transcripts at each stage is taken to represent both fully mature mRNA and precursor molecules. Therefore, these transcripts were characterized using Northern gel blot analysis of total cellular, cytoplasmic and polysomal polyadenylated RNA employing various DDC probes (see Figure 8 for probes).

Late Embryogenesis

The transcripts associated with the DDC activity peak in late embryogenesis were analysed first as their relationship to the transcripts during pupariation was of initial interest. In 14 hr embryonic total cellular RNA samples, six distinct transcript classes ranging from 2.0kb to 4.5kb in length (Table 3) can be visualized when gel blots are hybridized to the 4.8kb *EcoR1* DDC probe (Figure 15B,C). In Northern gel blots which are overexposed for these main bands, three more transcripts are apparent between the 4.5kb and the 3.25kb transcripts (Figure 17). The median sizes are 4.1kb, 3.8kb, and 3.5kb when compared on a standard curve to the other DDC transcripts (Table 3).

Thus, this collection of nine transcript size classes probably represents both fully mature translatable mRNA and its processing precursors.

The doublet consisting of the 2.0kb and the 2.15kb RNA transcripts probably represents the fully processed mature DDC mRNA. Both species are found associated with large molecular weight polysome structures (Figure 27) indicating that both seem to be translated. The hypothesis I favour is that the 2.15kb transcript gives rise to the 2.0kb molecule, probably through a variation in the length of the polyadenylation at the 3' end. The poly(A) tail is known to decrease in length during the life of a mRNA transcript (Brawerman, 1981). This variation has been shown to occur in discrete size classes for the globin mRNA (Gorski *et al*, 1974). In the surf clam *Spisula* this deadenylation event has been associated with translation (Rosenthal *et al*, 1983). In both cases mentioned the length of the poly(A) tail remains long enough (20) to remain in the poly(A)+ fraction from oligo-dT chromatography (Taylor, 1979), similar to the observation for both DDC transcripts. Both contain enough sequence information to comfortably contain the 1.6kb approximate coding length, (assuming the average M.W. of an amino acid is 100 daltons), required to produce the 54,000 dalton protein subunit of the larval DDC enzyme (Clark *et al*, 1978), plus additional sequence for the 5' and 3' nontranslated regions and the 3' poly(A) tail found in most eukaryotic mRNA (Brawerman, 1974). Also, both the 2.0kb and the 2.15kb transcripts do not contain sequences complementary to the 0.6kb *EcoR1-BamH1* partial 5' intron probe (Figure 22). This 0.6kb *EcoR1-BamH1* DDC subclone is considered to represent intron sequences because DNA sequence information reveals that all three reading frames of the mRNA produced are closed by one or more nonsense codons (R.B.Hodgetts, personal communication). Thus it seems likely that the 2.0kb and the 2.15kb transcripts both represent fully spliced translatable DDC mRNAs and the variation in size is likely due to a deadenylation of the 3' end, which occurs during translation.

All other mRNA transcripts larger than the 2.15kb molecule presumably represent incompletely processed precursors. The fact that these transcripts are polyadenylated is consistent with the finding that polyadenylation precedes splicing in the cases where poly(A) mRNA is produced (Nevins and Darnell, 1978; Tsai *et al*, 1980).

In most systems studied to date splicing precursors have been found only in the nucleus (Ryffel *et al*, 1980; Tsai *et al*, 1980). However, splicing intermediates have been found in the cytoplasm from Adenovirus-2 transcripts (Nevins, 1979; Berget and Sharp, 1979) but it is not clear whether these transcripts are processed further, or are products of abortive splicing events, or are the event of nuclear leakage during experimental manipulation. In both cytoplasmic and polysomal RNA preparations minor amounts of the precursor transcripts are visualized (Figures 24 and 27). However, when compared to total cellular RNA preparations containing both nuclear and cytoplasmic RNA, the minor amounts of these transcripts in the cytoplasmic preparations clearly represent only a subset of the total cellular precursors. Their presence in these cytoplasmic RNA samples was confusing as most RNA processing is thought to occur in the cell nucleus. After obtaining this result an effort was made to determine if other investigators had been faced with similar results. Lenk *et al* (1977) have shown that the use of the non-ionic detergent NP-40 in cellular extraction buffers above 0.1% causes the solubilization and loss of the nuclear membrane. Along with this a visible change in the morphology of the chromatin occurs but the nucleus remains intact. Thus, the nuclear skeleton is sufficient to maintain structural integrity but cannot prevent nuclear leakage. Therefore, the low level of precursors visible in the cytoplasmic preparations is probably due to nuclear leakage caused by the 0.5% NP-40 in the extraction buffer.

Additional evidence that these transcripts are precursors comes from their hybridization to the 0.6kb *EcoR1-BamH1* intronic probe (Figure 22) mentioned above. Also initial hybridization results indicate that the 0.3kb *Xho1-HindIII* DNA fragment is complementary to only the 4.5kb transcript. The restriction map (Figure 8) shows this fragment lies within the 3' intron. This, in conjunction with sequence data (R.B. Hogetts, personal communication) showing stop codons in all three reading frames of this DNA fragment, suggests that it represents intron sequence. Thus the hybridization results with these two intron probes show that these transcripts contain genomic sequences not found in the 2.0kb and the 2.15kb mature transcripts.

During maturation of the ovomucoid (Tsai *et al*, 1980) and of the mouse major β-globin (Kinniburgh and Ross, 1979) primary transcripts, a preferred order of intron excision has been recognized; however in other cases no order of intron excision has been observed (Breathnach and Chambon, 1981).

The hybridization patterns of both DDC intron probes suggest that there is a preferred order of intron splicing during the maturation of the DDC primary gene transcript. The hybridization of the 0.6kb *EcoR1-BamH1* probe to all precursor molecules (Figure 20) and the initial result showing that only the 4.5kb transcript contains sequences complementary to the 0.3kb *Xho1-HindIII* probe (data not shown), can be interpreted to indicate that the 3' intron is removed first followed by the excision of the 5' intron sequences. Thus the 4.5kb transcript probably contains both complete intron sequences. The 3' intron, complementary to the 0.3kb *Xho1-HindIII* probe, is removed first to give rise to the 3.25kb transcript which then contains only 5' intron sequences. This 3.25kb transcript is then processed further to yield the 2.15kb fully spliced mature DDC mRNA.

The removal of intron sequences by multiple excision steps has been observed in a number of different systems. Many intermediates have been identified in the splicing pathway used to link the Adenovirus-2 major late tripartite leader sequence to the main bodies of the late messenger RNAs (for review see Ziff, 1980). RNA transcripts with partially excised introns have been visualized by R-loop mapping of Adenovirus-2 transcripts by Chow and Broker (1978). The chick α-2 procollagen mRNA is produced from a precursor transcript, from which intermediates with partially excised introns have also been identified (Avvedimento *et al*, 1980). The large intron from the mouse β-globin gene primary transcript requires at least 2 steps to be excised completely (Kinniburgh and Ross, 1979). However, in most RNA transcript pools these partially spliced intron transcripts are not easily identified. Kinniburgh and Ross (1979) believe that this under-representation of these splicing intermediates in the steady state RNA population is due to a combination of two factors. The initiation of intron excision may be the rate-determining factor of the splicing process, causing a build-up of molecules containing complete intron sequences. Once this barrier has been overcome the excision of the intron is relatively rapid, occurring in a number of steps giving rise to the fully spliced RNA transcript. Thus, this situation would cause these splicing intermediates to be only a minor fraction of the steady state RNA population and is probably the reason why these transcripts are not reported in the early literature dealing with RNA splicing precursors (Kinniburgh and Ross, 1979).

Considering this, the number of DDC transcript precursors and the variation in their steady state levels both suggest that the removal of the intron sequences from DDC transcripts occurs by multiple excision:religation events (see Figures 15B, 15C, 17, and 22). Also noticeable is that the precursor bands are of two types with respect to band width. The 4.5kb and the 3.25kb precursor transcripts are found in bands with little size variation which produce narrow intensely hybridizing spots in a gel blot autoradiogram. These transcripts are thought to represent molecules containing complete intron sequences. This is in keeping with the Kinniburgh and Ross (1979) hypothesis. The 4.1kb, 3.8kb, 3.5kb, and the 2.8kb and 2.3kb transcripts are all characteristically less intense than the transcripts flanking them. Also the bands are wider, indicative of a greater variation in the size of the molecules within them. This size variation ranges from about 100 to 150 nucleotides. These transcripts, considering their steady state prevalence, probably represent splicing intermediates containing only subunits of the complete introns. Thus, the splicing together of exonic coding sequences for the DDC transcript can be interpreted to take place by a number of excision:religation events to finally yield the properly joined exons.

The size variation of these bands, presumably containing partially spliced intron transcripts, may reflect a functional imprecision of the excision:religation event during the first few steps of intron removal. Thus each excision:religation event, removing 300-500 bases of intron, produces partially spliced transcripts of variable sizes, presumably due to the imprecise nature of these events. This seems probable as only during the final excision:religation event does the splicing process require a precise junction to be formed for the production of a functional mRNA.

Models for RNA splicing have been proposed as the accumulation of information about this process continues. Sharp (1981), has proposed that the nature of the splicing event is progressive, starting at a single site and working in either or both directions until the intron is fully removed utilizing multiple excision events. This explains how the process never splices the 5' site of one intron to the 3' site from another, deleting exonic regions in the process. Lerner *et al* (1980) and Rogers and Wall (1980) have both proposed that the small nuclear RNA U1 may be involved in the precise positioning of the 5' and 3' splice sites for the exact splicing of exonic regions due to the extensive sequence complementarity of this snRNA and the regions around the 5' and 3' splice junctions. Considering this, Sharp also proposed that the signal guiding the RNA splicing complex to bind to the intron is probably similar to that used by the ribosome to bind to the 5' end of an mRNA molecule. Recently, Langford and Gallwitz (1983) have identified a yeast intron-specific octanucleotide sequence, found in the 3' end of the yeast actin gene intron, which is absolutely required for its removal. These authors believe this sequence may represent the intron recognition element which allows the positioning of the RNA splicing complex. It seems possible that this type of sequence element may prove to be a general phenomenon of all intron sequences, however, the investigations are still to be done. Mount *et al* (1983) have also recently shown that the snRNP containing the small nuclear RNA U1 can selectively bind a 5' splice site *in vitro*. This suggests that the snRNA U1 could be involved in the exact positioning of the adjoining exonic regions prior to the final excision:religation event as proposed by Rogers and Wall (1980).

Thus from this information one could imagine that the splicing of the intron sequences from DDC transcripts occurs first by the binding of the splicing complex to some intron identification sequence, perhaps a small nucleotide region of a splice junction, then in a progressive fashion, the imprecise excision:religation of 300-500 nucleotides of intron region gives rise to a variation in the size of splicing intermediate so generated. This would continue until the complete intron is excised and the adjacent exonic regions are somehow accurately joined. This mechanism allows introns of various sizes and sequences to be identified and then removed accurately with little chance of deleting exonic regions.

The results from the 14 hr polysome gradient (Figure 27) show that the DDC precursor transcripts are all of the 80 S size class. These mRNA:protein complexes may represent splicing complexes. Kinniburgh and Martin (1976) have shown that most if not all of the ascite cytoplasmic poly(A) RNA represented in the nuclear polyadenylated RNA is associated with a 30 S protein complex composed of several different protein subunits. Beyer *et al* (1981) have also shown that most of the nascent RNA transcripts found in chromatin spreads of *Drosophila* embryo nuclei are associated with a number of 30-40 S monomer protein particles. These particles have been proposed to be responsible for many functions during RNA maturation including RNA cleavage. It would be interesting to test these 80 S DDC transcript mRNPs for the presence of snRNAs. More investigation is needed to determine the nature of these 80 S particles and their relationship to the splicing process.

Finally, the 14 hr polysome gradient shows a significant accumulation of the 2.3kb transcript in the same gradient fraction containing the translated DDC mRNAs (Figure 27). It is unclear from this result whether the presence of the 2.3kb transcript in this large polysome fraction is due to translation of this mRNA or whether its position in the gradient is due to the interaction of some other large macromolecule. Because this transcript still contains intron sequences, it is hard to believe that it is associated with ribosomes and is being translated. Firstly, Martin and Miller (1983) have shown that most mRNAs being translated are completely saturated with ribosomes along their entire length. The position of this transcript in the polysome gradient indicates that if it is associated with ribosomes, similar numbers are found on this and the other mature DDC transcripts. DNA sequence data has shown that this 5' intron contains multiple stop codons, therefore, unless all have been precisely removed no ribosomes could transit past this region of the transcript making its migration into the large polysome gradient fraction due to some other factor. However, if all stop codons are successfully removed, and the extra nucleotide sequence does not alter the frame of translation, this extra intron sequence would be translated into additional exonic regions, making the protein produced larger than that made from the 2.15kb and 2.0kb transcripts. This would be similar to the situation that occurs for the αA-crystallin gene described by King and Piatigorsky (1983). Alternatively, if the extra nucleotide insert altered the frame of translation one might expect a smaller protein product caused by premature translation termination. Initial results indicate that a 54,000 dalton protein subunit is seen in the immunoprecipitates from *in vitro* translation products from the late embryonic mRNAs (Figure 29). A slightly larger protein is not evident in this gel. However, due to the overloading of the gel with IgG protein and the severe problem of non-specific binding of smaller proteins to the immunoprecipitates, further investigation is necessary before these possibilities may be discounted. I feel that the best explanation for the presence of the 2.3kb DDC transcript in this polysome gradient fraction is this is due to an artifact of the extraction procedure caused by NP-40 in the extraction buffer. As mentioned earlier, this non-ionic detergent solubilizes the nuclear membrane that allows leakage from the nuclei. Perhaps the sedimentation coefficient of this 2.3kb complex is due to the association of the 80 S mRNP with a nuclear transport matrix or splicing complex, which upon cellular disruption is solubilized into the extract supernatant. Recently, Peebles *et al* (1983) have shown that the yeast tRNA splicing endonuclease activity is associated with the yeast extract membrane fraction, which can be solubilized by treatment with non-ionic detergents. More experimentation is needed to establish the nature of this 2.3kb mRNP. The sensitivity of the 2.3kb mRNP's molecular weight to treatment with EDTA may provide some insight into the nature of its components and their interactions. Further investigation should analyse the bouyant density of the large mRNP particles in metrizamide gradients using the methods of Ballinger and Pardue (1983). This will permit the differentiation of mRNP:macromolecular complexes from polyribosome complexes of different densities.

It is clear that numerous interpretations of the data are possible. The model present below is only intended to provide a working hypothesis to direct empiracle falsification. Thus, from literature precedent and the available data, the following model for the maturation of the DDC primary transcript is proposed. The 4.5kb transcript represents the polyadenylated primary gene transcript produced first at about 12 hr of embryonic development. This molecule contains both the 5' and 3' introns intact. The first maturation step is the removal of the 3' intron, of which the initiation step of the splicing process is rate-determining. After splicing initiation has taken place this intron is rapidly removed in a number of excision:religation events to give rise to minor steady state amounts of the intermediate splicing products which are 4.1kb, 3.8kb and 3.5kb in size. The initial excision of intron sequences presumably starts from a single point within the intron and progressively removes 300-500 nucleotides with each event. This brings the two splice junctions closer together until the final splicing event precisely joins the two adjacent exonic regions according to some guiding mechanism. The complete excision of the 3' intron gives rise to the 3.25kb transcript. Again, the rate determining nature of the splicing initiation step for the removal of the 5' intron causes this transcript to have relatively high steady state levels. The removal of this intron also occurs by multiple excision:religation events giving rise to the 2.8kb and the 2.3kb splicing intermediates and finally the 2.15kb fully spliced DDC mRNA. This molecule is exported from the nucleus and then translated to give rise to the 54,000 dalton DDC subunit. During translation the polyadenylated 3' end of the 2.15kb molecule is shortened to give rise to the 2.0kb transcript. This transcript then becomes non-polysomal and finally degraded.

Further investigation should focus on the sequence characterization of all the transcripts found during 14 hr of embryonic development. This may be accomplished using the S1 hybridization protection assay of Berk and Sharp (1977), however the results using this method could prove difficult to interpret, due to the complex mixture of transcripts in these RNA preparations. Alternatively, the plasmid-mediated cDNA cloning method of Okayama and Berg (1982) which allows a high proportion of full length cDNA clones to be isolated, may be employed. The isolation of cDNA clones made from size-fractionated mRNA (Figure 32) will allow the sequence differences between transcripts to be determined.

Early Embryogenesis

The significant amount of transcript found during early embryogenesis and in ovaries from virgin females (see Figures 13 and 15) was surprising, as very little or no DDC activity can be seen at this time during development (Figures 2-4). This 2.2kb transcript differs in size

(Figure 21) and hybridization response to the 0.6kb *EcoR1-BamH1* intron probe, (Figure 22) indicating that it is qualitatively distinct from any at 14 hours of development. The presence of this transcript in both the 1 hr and 2 hr embryo samples as well as in developing ovaries suggests that it may be supplied during oogenesis to the oocyte as a maternal mRNA by the nurse cells of the developing oocyte follicle. This 2.2kb transcript is probably not produced by transcription from the zygotic nucleus after fertilization, as it has been shown by several laboratories that very little if any transcription occurs from the embryonic nuclei until after cellular blastoderm (Lamb and Laird, 1976; McKnight and Miller, 1976; Zalokar, 1976; and Anderson and Lengyel, 1981). Syncytial blastoderm in the developing embryo occurs at about 2 hours of development in this collection and incubation scheme (Figure 14).

Maternal RNA in other insects is found as a RNA:protein complex (Paglia *et al*, 1976) as originally observed by Spirin (1969) in developing fish embryos. The size of these particles in *Drosophila* range from about 10 to 80 S or more (Goldstein, 1978). These maternal mRNA molecules are thought to be responsible for initial development after fertilization. In sea urchins this maternal mRNA can direct embryonic development through many cleavages in the absence of nuclear gene expression (Gross and Cousineau, 1964).

Initial studies in *Drosophila* by Goldstein and Snyder (1973) and Lovett and Goldstein (1977) have shown that after fertilization there seems to be an increase in polysomal translation. This suggested to these authors that the mRNP complexes could be acting as a general mechanism for inhibiting the translation of stored maternal mRNA until after oocyte fertilization. However, Mermod and Crippa (1978) have shown that the translation in the polysomes of oocytes differed little from that seen in fertilized embyros which agrees with the result of Zalokar (1976) in which unfertilized oocytes laid by virgin flies incorporated substantial amounts of radioactive amino acids into proteins. Recently Mahowald *et al* (1983) have shown that there is an increase in polyribosome formation and translation upon oocyte activation prior to fertilization. Thus it seems that unfertilized eggs become translationally activated prior to their fertilization.

Goldstein(1978) has also shown that during early embryogenesis the RNA found in the prepolysome and polysomal fractions of the sucrose gradients were qualitatively identical. This indicates that at the start of embryogenesis the majority of the mRNP molecules are released from the translational block. However, specific mRNPs still appear to be translationally controlled. Mermod *et al* (1980) have shown that some maternal mRNPs, coding for DNA binding proteins, are not translated until the third hour of embryogenesis. Jäckle(1980) also demonstrated that in *Smittia* maternal mRNPs coding for actin are not translated until much later in embryonic development. This actin production is insensitive to actinomycin-D, suggesting that it is due to the translation of these mRNP molecules. These examples suggest that translational control can act by regulating the expression of specific genes.

The majority of the 2.2kb DDC transcript found in the 1 hr and 2 hr embryo polysome gradients occurs in the prepolysomal fractions characteristic of non-translated mRNP molecules (Figure 26). The size of the particles ranges from approximately 10 to 80 S as determined by their migration in the sucrose gradient. Only a minor fraction of the 2.2kb transcripts are seen associated with the polysome portions in the 1 hr and 2 hr samples (Figure 26). The small amount of polysomal 2.2kb transcript in these early samples is likely due to the variation in sample age caused by the collection scheme. Thus considering this we can say that until about 2 hr of development these 2.2kb mRNPs are not being translated suggesting that their expression is regulated by specific translational control, similar to the examples described by Mermod *et al* (1980) and Jäckle (1980).

In the third polysome gradient (2-4 hr, Figure 26), representing a sample of embryos with a mean age between 2 and 3 hr, there seems to be a reduction in the amount of prepolysomal 2.2kb DDC transcript. The polysome fractions of this sucrose gradient now contain this 2.2kb transcript indicating that it may be associating with numerous ribosomes and is being translated. Also noticeable in the last fraction of 2-4 hr gradient (P4) is some 2.0kb sized transcript (Figure 26). This 2.0kb transcript is also seen in the polysome fraction (P3) of the 2 hr polysome gradient (Figure 26). In Figure 15C, minor amounts of this presumed 2.0kb transcript are visualized in most early embryo samples but at 3 and 4 hr of development there seems to be an apparent increase in the steady state level of this molecule. Significantly, no 2.2kb transcript can be detected beyond 2 hr of development. Thus, this may be taken to indicate that the 2.2kb transcript may be acting as a precursor to the 2.0kb transcript and this conversion takes place after the recruitment of the 2.2kb molecule to form polyribosome structures. This processing of the 2.2kb transcript to the 2.0kb transcript is probably involved with the shortening of the 3' polyadenylated tail, similar to that hypothesized to occur at 14 hr of development. Thus, from these results it seems that the 2.2kb mRNA is a maternally contributed transcript put into the oocyte prior to the completion of oogenesis. The majority, if not all of the 2.2kb mRNPs, are non-polysomal, until between 2 hr and 3 hr of development when these mRNP molecules are recruited into the polysomes and translated. This translation gives rise to a deadenylation of the 3' end to produce the 2.0kb transcript which can be seen to increase in concentration at 3 hours. The translation of these transcripts (the 2.2kb and the 2.0kb) is probably responsible for the minor increase in DDC baseline activity seen at about 3 hours of development (Table 4).

If this is the case, what purpose could this early embryogenesis DDC activity serve? Schlaeger and Fuchs (1974) have described an increase in DDC activity, found in the oocyte during the later stages of oogenesis, which is induced by a blood meal in the mosquito *Aedes aegypti*. These authors believe this rise in DDC activity is required to sclerotize the egg chorion shortly after oviposition. Perhaps this early embryonic DDC activity has an analogous role in *Drosophila melanogaster*.

The other transcripts seen at this time of development (Figures 14C and 20) presumably represent precursors. These transcripts are found to be about 5.2kb, 4.5kb, and 3.25kb in size (Table 3). The 4.5kb and 3.25kb transcripts are presumably similar in sequence to the 14 hr RNA transcripts previously described. Of considerable interest is the appearance of the unique 5.2kb transcript. The increased size of this transcript opens the possibility that during the production of the 2.2kb transcript in oogenesis, the initiation of transcription at the DDC gene begins at a novel mRNA start site. Thus the transcription of the DDC gene at this time may occur from a distinct 5' promoter element. The 50 nucleotide increase in the size of the 2.2kb transcript compared to the 2.15kb 14 hr transcript supports the notion that a longer 5' mRNA leader sequence has been added to this transcript, similar to that described by Benyajati *et al* (1983) for the ADH gene in *Drosophila melanogaster*. Thus, the 5.2kb transcript may represent the primary gene transcript produced during oogenesis from an upstream mRNA transcription start site about 700 bases 5' to the initiation site used during late embryogenesis. The 4.5kb transcript is produced by the splicing of this new leader sequence into place. The introns are presumably spliced out next, giving rise to the 3.25kb and finally the 2.2kb transcript.

The appearance of these precursor molecules in the early embryo samples and not in the ovary RNA is probably a function of the mechanism of their contribution to the oocyte. Most if not all of the polyadenylated mRNA found in the cytoplasm of the unfertilized oocytes of *A. polyphemus*, is contributed by the nurse cells during oogenesis (Paglia *et al*, 1976). During the later stages of oogenesis in *Drosophila melanogaster* the nurse cells also contribute various macromolecular components into the cytoplasm of the developing oocyte as they degenerate (King, 1970). Perhaps the 5.2kb, 4.5kb, and the 3.25kb transcripts represent molecules contributed to the maturing oocyte after the nurse cells have lost the ability to process them into the 2.2kb mature transcript. The increased amounts of the 5.2kb transcript over the other two precursor molecules suggests that a block in the processing occurred prior to the exclusion of nurse cells from the developing oocyte. The reason there are no precursors evident in the ovary RNA sample is probably because dissected ovaries contain a great variation in the stages of developing oocyte that are represented, with very few being similar to the newly fertilized egg. The persistence of these precursors until 4 hr of development may be due to the embryo's inability to degrade them until this time.

Thus, the 2.2kb DDC transcript found in oocytes and preblastoderm embryos seems to be contributed to the egg by the maternal genome prior to fertilization. The expression of this transcript seems to be regulated by a mRNP specific translational control mechanism that does not allow this transcript to associate with ribosomes until between 2 and 3 hr of development at 29°C. Further investigation into the translational control of DDC expression at this stage will no doubt provide valuable insight into the mechanism involved in translational control of maternal mRNP molecules. From these results it is possible that this ovary specific transcript may be produced using a different RNA start site up stream from that used later in development. Further investigation into the sequence characterization of this RNA transcript, as was proposed above for the late embryonic transcripts, is important to begin analysing the factors regulating DDC gene expression at this stage.

Mid Embryogenesis

The function of the minor amounts of mature and precursor transcript seen between 4 and 10 hr of development is not understood (see Figures 13, 15 and 22). Perhaps these transcripts are due to a small percentage of sample asynchrony, due to females not immediately depositing fertilized eggs. Alternatively, these mRNAs may represent the production of DDC specific to the nervous system in *Drosophila melanogaster*. Recently, Livingstone and Tempel (1983) have shown that DDC activity is responsible for the production of the neurotransmitters dopamine and serotonin in adult *Drosophila* brains. Thus this low level of constitutive DDC gene transcription could represent the production of neuronal enzyme. Fullilove and Jacobson (1978) have stated that the neuroblasts that eventually form the larval brain are recognizable as early as 4 hr after the beginning of development at 25°C. The localization of these mid-embryonic DDC transcripts within the embryo using the *in situ* hybridization techniques of Zimmerman *et al* (1983) may help clarify this point.

CONCLUDING REMARKS

Initial investigations into the characterization of the expression of the DDC gene during embryogenesis are presented. The expression of the DDC gene during late embryogenesis was shown to have a different temporal relationship to the ecdysteroids found at this stage when compared to that seen for DDC expression during pupariation as demonstrated by Kraminsky *et al* (1980) and W.C.Clark (personal communication). The exact nature of the interaction between the ecdysteroids and the DDC gene and the possible regulatory functions of these compounds at each stage is unknown at this time, however the situation during both embryogenesis and pupariation correspond well to the late and early puffing responses respectively, described by Ashburner (1974). The expression of DDC at each stage should be investigated with regard to the chromatin structure in and around the gene as reflected by the occurence of DNaseI hypersensitive sites using the methods of Stadler *et al* (1978). This type of analysis may provide some insight into the differences in the regulation of DDC gene expression at both stages. Also an effort should be made to investigate the interaction of the ecdysteroid receptor complex with regions of the gene promoter using the methods similar to those decribed by Mulvihill *et al* (1982) and Yamamoto *et al* (1982). These types of investigations will provide better understanding of the relationships the ecdysteroids have with DDC expression at each stage.

From the data presented in this thesis, a model for the splicing of the primary DDC gene transcript seen during late embryogenesis has been put forth. Further experimental investigation is necessary adequately test this hypothesis, including the establishment of transcript sequence information.

The expression of the DDC gene during early embryogenesis also requires further investigation. The possibility that the 2.2kb transcript produced during oogenesis and maternally contributed to the embryo is transcribed from a stage-specific promoter located about 700 bases 5' to the mRNA start site used during late embryonic expression, including the establishment of sequence information for these early transcripts, should be investigated further. The early embryonic 2.2kb DDC transcript also provides a system to study the mechanisms involved in translational regulation of expression used for maternally contributed mRNAs. Further investigation here may be helpful in understanding the mechanisms of specific translational control over gene expression. Thus, the study of DDC gene expression at both early and late embryonic stages may prove useful in analysing the controls over the flow of information from DNA to the final polypeptide product.

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